Development of multi-residue and selective methods for the ultra-sensitive determination of endocrine disrupting chemicals in aqueous samples

Els Van Hoeck

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Promotor: Prof. Dr. P. Sandra

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LIST OF ABBREVIATIONS

AA	Acrylamide
AAA	Acetic acid anhydride
ACN	Acetonitrile
AIBN	2,2'-azobis(isobutyronitrile)
Ala	Alanine
APE	alkylphenol polyethoxylates
APT	Attached proton test
Arg	Arginine
ATR	Atrazine
B(a)P	Benzo(a)pyrene
Bis	N,N'-methylene bisacrylamide
BPA	Bisphenol A
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
$CDCl_3$	Chloroform-deuterated
COSY	Correlation spectroscopy
DCM	Dichloromethane
DDA	Desethydesisopropylatrazine
DDD	Dichlorodiphneyldichloroethane
DDE	Dichlorodiphenyldichloroethene
DDT	Dichlorodiphenyltrichloroethane
DEA	Desethylatrazine
DIA	Desisopropylatrazine
DEHP	Di(2-ethylhexyl)phthalate
DES	Diethylstilbestrol
DIEA	N,N-diisopropylethylamine
DMF	N,N-Dimethylformamide
DMSO	Dimethylsulfoxide
DVB	Divinylbenzene

E2	17-β-estradiol
EA	Ethyl acetate
ECF	Ethyl chloroformate
EDCs	Endocrine disrupting chemicals
EDTA	Ethylendiaminetetra-acetic acid
EE2	17-α-ethinyl estradiol
ELISA	Enzyme linked immunoassay
EtOH	Ethanol
eq	equivalents
FT-IR	Fourier transform infrared spectroscopy
GC	Gas chromatography
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
h	Reduced plate height
HBD	Hormone binding domain
hER	human estrogen receptor
His	Histidine
HOAc	Acetic acid
HPLC	High performance liquid chromatography
IAE	Immuno-affinity extraction
ID	Internal diameter
Ile	Isoleucine
K_d	Dissociation constant
$K_{o/w}$	Partition coefficient between octanol and water
$K_{pdms/w}$	Partition coefficient between PDMS and water
LC	Liquid chromatography
LD	Liquid desorption
Leu	Leucine
LLE	Liquid liquid extraction
LOD	Limit of detection

LOQ	Limit of quantification
LVI	Large volume injection
MASE	Membrane assisted solvent extraction
MeOH	Methanol
Met	Methionine
MIPs	Molecularly imprinted polymer
MS	Mass spectrometry
MW	Molecular weight
$NaBEt_4$	Sodium tetraethylborate
NaCl	Sodium chloride
NaOAc	Sodium acetate
NH_3	Ammoniak
NH_4OAc	Ammonium acetate
NMR	Nucluar magnetic resonance
NP	4-n-nonylpenol
OD	Outer diameter
PAHs	Polycyclic aromatic hydrocarbons
PBrBs	Polybrominated biphenyls
PCBs	Polychlorinated biphenyls
PDMS	Polydimethylsiloxane
Phe	Phenylalanine
ppb	parts-per-billion (µg/L)
ppm	parts-per million (mg/L)
ppt	parts per trillion (ng/L)
Pro	Proline
PyBOP	(Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate
PTV	Programmed temperature vaporization
RAM	Restricted access material
RSD	Relative standard deviation (%)
Ser	Serine

SBSE	Stir bar sorptive extraction
SEM	Scanning electron microscopy
SFC	Supercritical fluid chromatography
SIM	Single ion monitoring
SLM	Supported liquid membrane extraction
SMSE	Silicone membrane sorptive extractionμ
S/N	Signal to noise ratio
SPME	Solid phase micro extraction
SPE	Solid phase extraction
S/SL	Split-splitless inlet
TBT	Tributyltin
TD	Thermal desorption
TDS	Thermal desorption system
TDU	Thermal desorption unit
Tes	Testosterone
TGA	Thermogravimetric analysis
THF	Tetrahydrofuran
TLC	Thin layer chromatography
UV	Ultra-violet detection
Val	Valine
VP	4-Vinylpyridine

PREFACE

Since World War II, the world has witnessed a large increase in the production of chemicals. Some of these chemicals, like pesticides, were designed for wide spread use. Others, like polychlorinated biphenyls (PCBs), were rather accidentally released into the environment by leakages or waste dumping. At that time, little or no attention was paid to the possible consequences that could result from the use, misuse and distribution of these chemicals.

This ignorance changed with the publication of Rachel Carson's *Silent Spring* in 1962. This book documented for the first time the detrimental effects of pesticides on wildlife. More specifically, the link was proven between egg shell thinning of birds and the pollution of the surrounding environment with dichlorodiphenyltrichloroethane (DDT) [1]. From that time, public awareness was born and grew ever since.

Only three decades later, a new disturbing event was signalled in an article by Theo Colborn [2]. He described the deleterious influence that certain chemicals can have on the development of endocrine systems. These endocrine disrupting chemicals (EDCs) were linked to reproductive problems like the decrease in fertility of bird. Since then, there is a growing scientific concern, public debate and media attention over the possible effects on wildlife and humans that may result from exposure to chemicals that have the potential of interfering with the endocrine system [3].

Establishing a causal relationship between the presence of EDCs in the environment and their possible effects on human health is a challenging quest. Not only are the EDCs chemically very heterogeneous, but they cause adverse effects at concentration levels as low as 1 ng/L. In addition, the environmental matrix is very complex. Therefore, sample clean-up en pre-concentration of the sample is necessary before analysis.

In this framework, the current work focuses on the optimization of the sample preparation in order to develop selective and multi-residue methods for the determination of EDCs in aqueous samples. In the first part of the work, a multi-

residue method is developed using sorptive extraction in combination with *in-situ* derivatization. Since the extraction of polar analytes is limited when using sorptive extraction with polydimethylsiloxane (PDMS), a novel type of sample preparation namely sorptive membrane solvent extraction, was developed and evaluated for the determination of atrazine and its polar metabolites in aqueous samples. Another attempt for the determination of polar analytes was done by preparing monoliths as extraction medium. Finally, a new selective artificial receptor for endocrine disrupting chemicals, based on the human estrogen receptor, was synthesized and evaluated using affinity chromatography and solid phase extraction (SPE).

Although this work merely tips the iceberg with regard to providing total insight in the analysis of EDCs in aqueous samples, it aims to present the reader a broad overview of the different analytical techniques that can be used, their corresponding shortcomings and possible solutions. Furthermore, this work aims at inciting other investigators and the governmental bodies to continue the research in this area; as large deficits remain especially with regard to environmental and public safety.

References

[1] R. Carson, 1962. Silent Spring. Mariner Books, Boston, 400 p.

[2] T. Colborn, F.S.V. Saal, A.M. Soto, Environ. Health Perspect. 101 (1993) 378.

[3] P. Matthiessen, Pure Appl. Chem. 75 (2003) 2197.

CHAPTER I
ENDOCRINE DISRUPTING CHEMICALS

In the last decade, the increasing distribution of endocrine disrupting chemicals (EDCs) in the environment became of worldwide concern. This anxiety is caused by the adverse effect of these pollutants on the endocrine system of humans and wildlife, even at levels as low as 1 ng/L. While the influence on the reproductive systems of several animals has been thoroughly documented, the effects on human health are still the subject of intense debate.

In this chapter a brief overview is given of the mechanism of endocrine disruption and of the effects on wildlife and humans. Next, possible sources of exposure of humans and wildlife to EDCs are described. Finally, a summary of the different approaches for the detection of EDCs in aqueous samples is presented.

1 What are endocrine disrupting chemicals (EDCs)?

The International Programme for Chemical Safety (IPCS) has together with Japanese, USA, Canadian, OECD and European Union experts agreed on the following working definition for endocrine disruptors: [1]

> *An endocrine disrupter is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations.*

Currently, the European commission has listed 320 chemicals as possible EDCs. The list encompasses a variety of chemical classes, including natural and synthetic hormones, phytoestrogens, alkylphenols, bisphenol A, polyaromatic hydrocarbons (PAHs), polychlorinated and polybrominated biphenyls (PCBs, PBrBs), organotin species, phthalates, some pesticides and other chemicals. Each year more chemicals are added to this list [1].

Most of these chemicals are already regulated under existing legislation. For instance, the maximum contaminant levels for pesticides in drinking water are 0.1 µg/L, as is mentioned in the EU water quality directive [2]. A lot of other compounds are only suspected endocrine disruptors and further research is necessary, so that new legislation can be established.

2 Mechanism of endocrine disruption

The endocrine system is a complex network of glands, hormones and receptors. The endocrine glands, such as the thyroid, gonads and adrenal glands, are situated at various sites in the body. They secrete specific chemicals called hormones such as thyroxine, estradiol, testosterone and adrenaline. The hormones travel through the bloodstream and elicit specific responses in other parts of the body by interacting with their specific receptor. As a result, all the significant processes in the body, such as development, growth, reproduction, immunity and behaviour, are regulated. The hormone concentration can vary, but it must be maintained between an upper and lower limit (homeostasis), otherwise detrimental effects can occur.

Endocrine disrupting chemicals are believed to interfere with the functioning of this complex system in at least three possible ways:

- by mimicking the action of a naturally-produced hormones like estradiol or testosterone and thereby causing similar reactions in the body (agonistic affect);
- by blocking the receptors thereby disturbing the binding of natural hormones to the receptors (antagonistic effect);
- by affecting the synthesis, transport, metabolism and excretion of hormones, thus altering the concentrations of natural hormones

Most of the known disturbing effects originate from the binding of chemicals with the estrogen receptor [3].

The estrogen receptor belongs to the family of steroid receptors and is thus a member of the nuclear receptors. The steroid receptors consist of three functional domains. The first is a N-terminal which regulates hormone responses. The second domain is the DNA-bindings domain. It consists of two Zn^{2+} fingers and regulates the gentranscription. The last domain is a strongly hydrophobic C-terminal hormone binding domain (HBD) which is responsible for the binding of the steroid.

The human estrogen receptor exists in two isomers, referred to as hERα and hERβ. The concentrations of these two types of receptor are different throughout the

body as well as their biological role. Since the composition of both HBDs is very similar, most EDCs interact in the same way with both isomers.

The human estrogen receptor (hER) is unique in its ability to embrace a wide variety of non-steroidal compounds. This overall promiscuity can be ascribed to the large size of the hormone binding domain (HBD) which has an accessible volume (450 $Å^3$), twice the size of estradiol (250 $Å^3$) [4]. Consequently, a lot of chemicals can bind with the ER and thus influence the endocrine system [5].

3 Effects of endocrine disruption

3.1 Effects on animals

A direct effect of chemicals on the endocrine system of animals was observed in the 1980s with the alligator population of Lake Apopka, central Florida (USA). It was noticed that this population declined as a result of contamination of the lake with the pesticide DDE, while the populations in the rest of Northern America increased. The pesticide pollution was linked to reproductive abnormalities like a smaller penis size, lower plasma concentrations of testosterone and higher concentrations of estradiol within juvenile, male alligators [6].

Another notable and convincing biological response to the presence of EDCs, is the production of the female fish hormone vitellogenin in male fish. Although this hormone is normally produced by the yolk of the female, high values of it have been found in male fish in a variety of water bodies in Europe, Japan and North America. In the UK, domestic waste effluents are a major source of pollution of the rivers. The effluents are contaminated with EDCs which results in the feminization of male fish which is reflected in the production of vitellogenin [7].

Furthermore, EDCs can already cause an adverse effect at very low concentrations (< 1 ng/L). For example, the embryo production of snails was significantly higher when they were exposed to ethinyl estradiol with a concentration of 1 ng/L for 21 days [8].

These examples are only a few of the well documented effects on wildlife. The effect of EDCs on animals still raises a lot of concern since it reflects the overall contamination of the environment. Indeed, problems with wildlife can be regarded to as a warning for humans.

3.2 Effects on humans

The effect of EDCs on humans is mostly predicted by studying the effects on animals, although some significant conclusions can be drawn from the

diethylstilbestrol (DES) case. Diethylstilbesterol is a drug that was administered to several million women in the late 1930's to prevent habitual abortion but was banned later on with the discovery that some women exposed in utero developed a vaginal cancer (the DES daughters). Also an increased infertility and an irregular menstrual cycle were observed with the female offspring. Adverse effects on the male reproductive system from the in utero exposure to DES consisted in a decreased sperm count, increased incidence in abnormal sperm and smaller penises. Because of the wide spread use of this drug, a clear relationship could be established between the use of the endocrine disrupting DES and the effects on humans [9].

Nevertheless, there is a big difference between the high amounts of DES given to pregnant women and the low concentrations of EDCs present in the environment. DES was administered in high doses and is more active than endogenous estrogens while common pollutants with estrogenic activity are not only present in very low concentrations, they are also less active then the endogenous estrogens. Consequently, reports of low-dose effects of exogenous EDCs are highly controversial and subject of intense debate [10]. Nevertheless, the exposure of humans to EDCs still generates concerns. This is because analysis of human data shows that there are human health effects in which EDCs can play a significant role. With males in particular, the decrease in the sperm count/quality and the increase in testicular cancer have been linked to the exposure of estrogenic chemicals during the foetal development [11, 12]. For women, it has been proposed that menstruation and sexual maturation (early puberty) occur now at lower ages than before as a result of the exposure to EDCs. Another effect that has been dedicated to EDCs is the steadily increase in breast cancers over the past decades in different countries [13].

One of the reasons why it so difficult to establish causal relationships between EDCs and their effects is that the effect can vary depending on the time of exposure. For example, exposure to EDCs during fetal and post-natal life can result in permanent changes since the endocrine system is programmed during that period. When adults are exposed to the same EDCs, the effect will be much smaller, because it can be corrected by the homeostasis mechanism [14]. In addition, the magnitude of

the disruption depends on so many factors like for example the type of chemical, duration, frequency and route of exposure.

In conclusion, the effects of EDCs on the human health are not well documented at this moment. More information is needed to accurately quantify the human burden of hormonally active environmental chemicals [15].

4 Exposure to endocrine disrupting chemicals

There are numerous chemicals present in the environment that can disturb the endocrine system. These include synthetic and natural hormones, phyto-estrogens and industrial chemicals (insecticides, household detergents, etc).

Natural and synthetic hormones

Because of the use of estrogen-replacement therapy and livestock manure, many natural and synthetic estrogens have been released in the environment. As a result, many estrogens have been detected in the rivers, reservoirs, lakes and other waters [16, 17].

Women excrete 10 -100 µg of estradiol (E2), ethinyl estradiol (EE2), estrone (E1) and estriol (E3) daily during their menstrual cycle [18]. During the pregnancy, they secrete up to 30 mg of estrogen (mainly E3) daily [19]. The structures of these estrogens are given in **Figure I.1**.

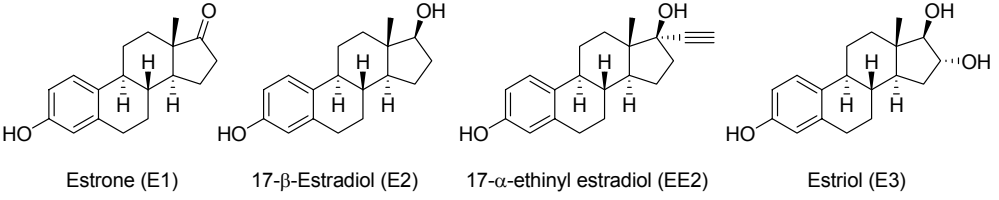


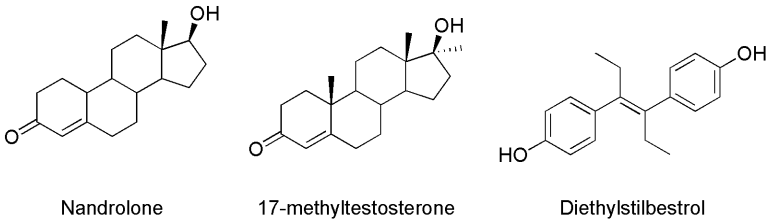
Figure I.1: Structures of the steroid hormones.

The presence of EE2 in the urine of women is the result of the use of EE2 in birth control pills. It is also used in hormone replacement therapy for post-menopausal women or in the treatment of breast cancer. EE2 is an example of a synthetic estrogen that is designed to have a greater estrogenic potency than the natural estrogens.

Animals also excrete natural estrogens. An estimated 10 million cows and 43 million pigs excrete a daily mix of 10-30 kg of 17-α-E2 in the US [20].

The pollution caused by natural and synthetic hormones has also increased due to the use of anabolic steroids as growth promoters to fatten cattle [21]. A few examples

of anabolic steroids are nandrolone, diethylstilbestrol and 17-methyltestosterone [22]. Their structures are given in **Figure I.2**



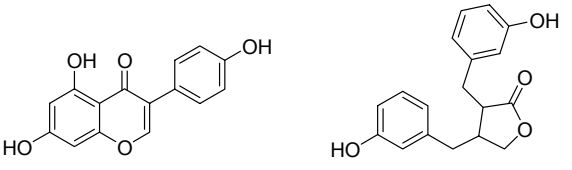
Nandrolone 17-methyltestosterone Diethylstilbestrol

Figure I.2: Structures of anabolic steroids used to fatten cattle.

The treatment with anabolic steroids may result in hormone residues in the meat, which could be harmful to the consumer [23]. Steroid hormones have also been found in fish [24] and poultry [25] and other animal-derived products such as eggs [24] and milk [26].

Phyto-estrogens

An important group of steroid look-alikes are the phyto-estrogens. They are a diverse group of naturally occurring non-steroidal compounds that are present in grains, vegetables and soybeans. They can be divided in two major classes. To the first class belong the flavonoids, more specifically isoflavones. An example of this class is genistein (**Figure I.3**), which is commonly found in soybeans, tofu and red clover. The lignans are the other major group of phyto-estrogens. An example of this group is matairesinol (**Figure I.3**), which occurs in numerous foods such as grains, fibres and fruits.



Genistein Matairesinol

Figure I.3: Structures of the most common phyto-estrogens.

The effect of exposure to these phyto-estrogens is not very clear. On the one hand, it has been show that they have some beneficial effects on human health, such as in

the prevention of cardiovascular diseases, osteoporosis and some cancers [27]. For example, the reduced risk of breast cancer in women in Singapore was found to correlate with a daily, high soy intake [28]. On the other hand, studies on animals have shown that phyto-estrogens can act as endocrine disruptors. Probably, the time of exposure is very important and that is why particular attention is given to babies who are fed with soy-based infant formulas [29].

Industrial chemicals

Man-made chemicals comprise thousands of new chemicals which are designed for use in industry, agriculture and consumer goods and which, apart from the uses for which they were designed, may have unforeseen endocrine disrupting effects. The hormonal activity of these chemicals is many times weaker compared to the natural occurring hormones like estradiol. For example, the estrogenic activity of nonylphenol, which is a breakdown product of alkylphenol ethoxylate surfactants, is only one thousandth of that of the natural hormone estradiol [5]. Nevertheless, some compounds are very lipophilic and have the tendency to bio-accumulate throughout the food chain, leading to higher concentrations and thus severe distortion of the endocrine system.

Pesticides

One class of chemicals that were released intentionally into the environment is the chlorinated aromatic insecticides like the notorious dichlorodiphenyltrichloroethane (DDT). Commercially available DDT is a mixture of *p,p'*-DDT and *o,p'*- DDT. (**Figure I.4**). A minor fraction consists of DDE and DDD, which are also the breakdown products of DDT [30].

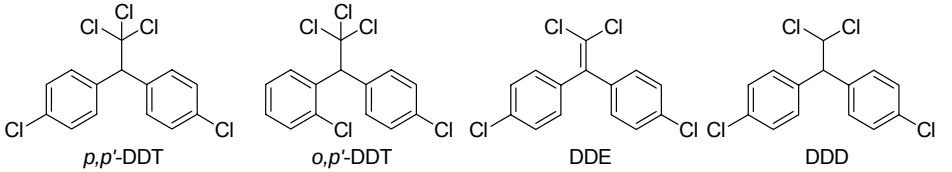


Figure I.4: Chlorinated aromatic insecticides.

In the second half of World War II, DDT was used on a large scale against mosquitoes spreading malaria and lice transmitting typhus, resulting in dramatic decrease of both diseases. From 1950 till 1980 DDT found wide spread use as a general insecticide in agriculture. After the publication of Rachel Carson's *Silent Spring* in 1962, it became clear that the release of large quantities of DDT in the environment had enormous consequences on wildlife and humans [1]. Hence, it was banned from world wide agricultural use. Although an exception was made for the fight against malaria in developing countries, the use of DDT remains controversial.

The chlorinated aromatic insecticides are very hydrophobic, persistent organic molecules. For example, a study in 2002 found detectable levels of DDT and its metabolites in the blood of more than half of the subjects tested [31]. This was the direct consequence of the past world wide use of DDT. As a result, the detrimental effects caused by these insecticides, are still noticeable. In vitro binding assays showed that the most estrogenic activity was displayed by *o,p'*-DDT [32]. Several studies were undertaken to determine a relationship between DDT levels in adipose tissue and breast cancer, but failed to find a significant link [5]. However, concentrations were determined in the patients at the moment they already developed the cancers, but maybe the exposure during foetal development and childhood plays a more important role.

After the discovery of DDT as an effective pesticide, large amounts of other pesticides like the organophosphates (e.g. chlorpyrifos), the carabamates (e.g. aldicarb) and the pyrethroids (e.g. cypermethrin), were developed and their use became widespread [33]. This period (1940s and 1950s) is considered to be the start of the "pesticide era" [34]. Since these pesticides are in general very old, insects have been exposed to them for many years and in many cases they are not nearly as effective as they used to be. As a consequence, other types of insecticides were developed such as the triazines (e.g atrazine) and even now research is still being carried out for the development of new pesticides. In general, pesticide use has increased 50-fold since 1950 and 2.3 million tonnes of industrial pesticides are now used each year [35]. Consequently, they form a major part of the class of endocrine disrupting chemicals.

Bisphenol A

Another threat is posed by estrogenically active chemicals that are unintentionally released into the environment like bisphenol A (BPA). The structure of BPA is given in **Figure I.5**.

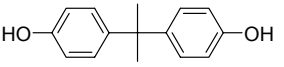


Figure I.5: Structure of bisphenol A

Bisphenol A is a frequently used monomer for the preparation of polycarbonates and epoxy resins. These polymers are used for plastic water bottles, baby bottles, plastic food containers and dental materials. The monomer BPA is known to leach from these products. For example, leaching of BPA occurs when baby bottles are heated. Consequently, infants fed with liquid formula can be exposed to BPA. A study by the European Food Safety Authority showed that daily intake of BPA for these infants can be as high as 13 μg/kg/day [36]. Although it was already shown in 1936 that bisphenol-A is an estrogenically active compound, only Canada intends to ban the use of BPA [37]. Europe and the USA conclude that there is a potential danger, but they state that current human exposure levels are too low to induce adverse effects. Nevertheless, the first bisphenol A free bottles are on the market to deal with growing public concern.

Phthalates

Phthalates in plastics are another source of estrogenic chemicals. These chemicals are called plasticizers because they soften hard plastics when added. They are not chemically bound to the polymeric framework and can thus migrate to the surface. Phthalates are found in soft toys, cosmetics, flooring, medical equipment and colourful prints on children's clothing. In vitro tests showed that they have weak estrogenic properties [38]. The most potent phthalate is bis(2-ethylhexyl)phthalate (DEHP). The structure is shown in **Figure I.6**.

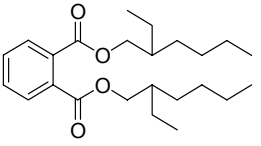


Figure I.6: Structure of DEHP

DEHP is used in medical tubing, catheters and blood bags. It may disturb the sexual development in male infants [39]. Based on these and other results, including animal testing, the European Commission has banned the use of phthalates in children's toys.

Alkylphenol polyethoxylates

Alkylphenol polyethoxylates (APEs) are non-ionic surfactants, used extensively in household detergents. This implies that they are disposed into the sewage system where they are broken down by micro-organisms (**Figure I.7**). The remaining alkylphenols are resistant to further biological degradation and appear in rivers [40].

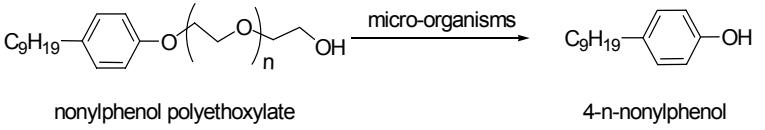
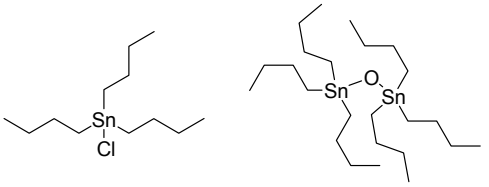


Figure I.7: Transformation of nonylphenol polyethoxylate to 4-n-nonylphenol.

Although there are many different APEs, 80% of them are composed from 4-n-nonylphenol (NP). This compound showed in vitro and in vivo estrogenic activity and is not only exposed to aquatic life, but also to humans via drinking water and consumption of fish [5]. The use of nonylphenol ethoxylates is now restricted by the European Commission.

Organotin compounds

Within the class of organometallic species, organotin compounds are probably the most widely spread in the environment due to their use as biocides in polymers, in the agricultural industry, as antifouling paints, etc. [41]. The most frequently used organotin compound is tributyltin (TBT) as tributyltinchloride or bis(tributyltin)oxide [42]. Their structures are shown in **Figure I.8**.



Tributyltinchloride bis(tributyltin)oxide

Figure I.8: Structures of the frequently used organotin compounds.

Alarming toxic effects on living organisms such as the masculinization of female snails (imposex) have been ascribed to the presence of TBT in aqueous systems [43]. The European commission issued the directive 2002/62/EG that prohibits the member states to put TBT on the market. Another law is in preparation to prohibit the use and presence of TBT on ships in the member states [44].

Polychlorinated biphenyls and dioxins

Polychlorinated biphenyls (PCBs) were used a coolants, insulating fluids for transformers and capacitors, stabilizing additives in flexible PVC coatings of electrical wiring and electronic components, etc. They are commercially available as mixtures like for example Aroclor 1260. The general structure of PCBs is given in **Figure I.9**.

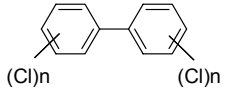


Figure I.9: General structure of PCBs.

In 1970 the production of PCBs was banned due to their high toxicity, persistency and their ability to bio-accumulate in animals. They may be destroyed by chemical, thermal and biochemical processes, though it is extremely difficult to achieve full destruction, and there is the risk of creating extremely toxic dioxins through partial oxidation. This is not the only source of dioxins. They can also enter the environment through burning of organic material in the presence of chlorine, so they are widely produced and in many contexts. The most toxic dioxin is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (**Figure I.10**).

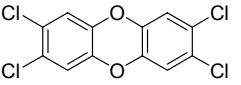


Figure I.10: Structure of the most toxic dioxin: 2,3,7,8-tetrachlorodibenzo(p)dioxin.

Dioxins mainly enter the general population by ingestion of food, specifically through the consumption of fish, meat, and dairy products. They are able to bio-accumulate, so even small exposures may eventually reach dangerous levels.

These examples show that we are exposed to EDCs on a daily basis in products that are used without suspicion. Careful monitoring of the concentrations and the potencies of the EDCs to which we are exposed is thus very significant.

5 Determination of EDCs in aqueous samples

The choice of analytical method for the determination of EDCs depends on the required outcome. Biological methods ascertain the endocrine disrupting activity exhibited by a chemical or sample, while chemical techniques identify chemicals and quantify their concentration within that sample. A combination of both approaches is necessary to identify the chemicals responsible for the estrogenic activity. Both techniques are briefly described.

5.1 Biological monitoring

Biological methods can be divided in two main categories, namely in vitro and in vivo assays. These are used to determine whether a chemical is an EDC.

In vitro assays are the first category of the biological methods. They are rapid, cost-effective tools requiring smaller amounts and they can achieve lower detection limits because of their specificity compared to chemical methods [45]. A few examples of in vitro assays are given here:

Receptor binding assays measure binding of agonists (or antagonists) to the human estrogen receptor (hER). These methods rely on the displacement of a high-affinity radioligand (^{3}H-labeled estradiol) by a possible estrogenic chemical. Its value is then compared to the displacement caused by the unlabeled estradiol and is expressed in relative binding affinities (E_2 being 100%). They have the feature of being very sensitive [46].

Cell proliferation assays depend on the ability of possible endocrine disruptors to induce rapid, uncontrolled cellular growth in the human breast cancer cell line MCF-7 [3]. These cells have high levels of estrogen receptors and consequently proliferation can be induced with very low concentrations of estrogenic substances.

Receptor-dependent gene expression assays measure the ability of a compound to stimulate a receptor-dependent response in genes. The most popular assay for the determination of estrogenic activity is the Yeast Estrogen Screen (YES assay). It consists of yeast cells in which the hER is expressed. When an EDC binds to the hER,

a reporter gene is transcribed and eventually translated into the enzyme β-galactosidase. This enzyme catalyses the hydrolysis of *o*-nitrophenol-β-D-galactopyranoside after which the concentration of the formed *o*-nitrophenol is determined via UV-measurements at 420 nm [47].

Enzyme linked immuno-assays determine the concentration of an EDC. The principle is based on the antibody-antigen interaction. The binding event is visualized using an enzyme which by binding to the analyte, transforms a colourless substrate into a coloured product. The concentration can then be measured using UV-spectroscopy [48].

These in vitro assays suffer from some drawbacks. The effects of bio-accumulation, metabolism and other possible pathways are not taken into account. Furthermore, there is poor correlation of the determined endocrine disrupting activity between different in vitro assays and their use is very limited in complex matrices [49].

In vivo assays are the other category of biological methods. A few examples are given here.

In the *uterotrophic assay* the ovary from mouse is removed, reducing the intrinsic female hormone levels to a minimum and thus shrinking the uterus. After administrating the test compound, the inflation of the uterus is monitored and the estrogenic activity of the compound is determined [10c].

The induction of *vitellogenin* production in juvenile male fish is another approach to measure the endocrine disruption activity of the aquatic environment [50].

Unfortunately, in vivo tests lack standardization which makes it difficult to compare results.

In conclusion, the in vitro and in vivo assays give complementary results and should be used together in a battery of tests [51].

5.2 Chemical monitoring

Whereas the biological approach identifies the estrogenic potency of a sample, chemical techniques identify the chemicals of interest and quantify their concentrations.

The chemical analysis of EDCs in environmental matrices is a difficult process not only because of the low concentrations at which the EDCs are present but also because of the complexity of the matrices (e.g. sewage water, serum, urine samples, etc.). Therefore, an extraction and clean-up procedure needs to be carried out before the sample is ready for analysis. An overview of the different sample preparation and analytical techniques for the determination of EDCs in water is given in Chapter II.

5.3 Combination of biological and chemical monitoring

Linking biological and chemical methodologies allows the determination of both the endocrine disrupting effect of a sample and the structural elucidation and concentration of the compounds responsible for this effect. In addition, the combination can also be used to increase the sensitivity and the selectivity. A few examples of possible combinations are given.

In *Immunosorbent SPE-LC-MS* monoclonal antibodies to E1 and E2 were utilized to produce an immunosorbent that was able to extract steroid estrogens. After elution, the extract was analysed with LC-MS [52].

Receptor affinity chromatography followed by LC-MS-MS uses immobilized ERs on a receptor affinity column. After loading of the sample and a washing step, receptor-relevant chemicals are eluted, that can then be identified and quantified by LC-MS-MS [53].

In conclusion, the best results are obtained when a biological method is used to evaluate a large number of samples, followed by chemical analysis of the samples that respond EDC positive.

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CHAPTER II

ANALYSIS OF EDCs IN AQUEOUS SAMPLES: SAMPLE PREPARATION IS THE KEY STEP

The analysis of endocrine disrupting chemicals is very challenging. Not only are the EDCs chemically very heterogeneous, but they cause adverse effects at concentration levels as low as 1 ng/L. In addition, the environmental matrix is very complex. Therefore, sample clean-up and pre-concentration of the sample is necessary before analysis.

In this chapter an overview is presented of the most common sample preparation methods for aqueous samples. Their use for the determination of EDCs is illustrated.

1 Introduction

The ideal scenario for trace analysis of pollutants in liquid samples is that the analytical technique chosen to perform the analysis requires no sample pre-treatment prior to introduction into the chosen instrument. However, reality is often very different. This can be due to the presence of particles (LC) or non-volatile sample constituents (GC), but most often the concentration of the analytes in the sample is simply too low. Especially in the analysis of environmental samples, enrichment is of vital importance because samples are too dilute and too complex for direct injection.

The quality of sample preparation is a key factor in determining the success of analysis and thus there is a considerable interest in developing new selective and sensitive methods for extracting and isolating components from complex environmental matrices. An ideal sample preparation technology should be fast, accurate, precise and should consume little organic solvent. Using 100 mL dichloromethane to extract semi-volatile analytes from a water sample may serve as a typical example of how not to proceed. Other demands for modern extraction methods include high throughput, compatibility with subsequent analysis and use of low cost materials. Finally, there is a need for enrichment techniques not only for hydrophobic compounds, but also for polar substances.

2 Liquid-liquid extraction

The principle of liquid-liquid extraction (LLE) is that the sample is partitioned between two immiscible solvents in which the analytes and the matrix have a different solubility. For example, an extraction can be obtained by shaking the aqueous phase containing the analytes with an organic phase such as hexane or dichloromethane. The main advantage of this approach is the wide availability of pure solvents and the use of low-cost apparatus. This technique suffers from some major drawbacks. Firstly, large amounts of toxic organic solvents are needed for the extraction of very small amounts of pollutants. Secondly, additional clean-up steps are necessary. Another drawback is the possible formation of emulsions when the immiscibility of the two phases is insufficient. A last disadvantage is that the extraction is time consuming.

Notwithstanding all these disadvantages, LLE has already been used by Korenaga et al. for the extraction of alkylphenols and bisphenol A in environmental water samples [1]. LLE was carried out using a water sample with a volume of 1 L and two times 50 mL dichloromethane. The water sample was first acidified to pH 2 and saturated with salt. After the extraction, the organic phase was evaporated to dryness and re-constituted in 500 µL acetone. The derivatizing reagent N,O-bis(trimethylsilyl)trifluoroacetamide was added to sylilate the phenolic functionalities. Finally, GC-MS analysis was carried and the obtained limits of detection were 1 µg/L

The amount of organic solvent used for the extraction could drastically be reduced with the use of a membrane for extraction of organic compounds. Hauser and coworkers introduced membrane-assisted solvent extraction (MASE) for the enrichment of hydrophobic compounds. In this technique, hydrophobic organic compounds are extracted through a non-porous polypropylene membrane into a small volume of organic solvent (ca. 800 µL) [2]. The set-up is demonstrated in **Figure I.11**.

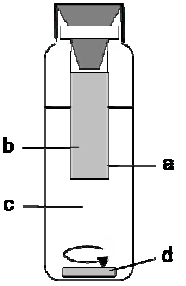

Figure I.11: Set-up of MASE: Polypropylene bag (a), organic solvent (b), aqueous solution (c) and a stirring bar (d).

The organic solvent should have a low solubility in water in order to minimize solvent loss via passage through the membrane. After the extraction, the organic phase can be analyzed with large volume injection (LVI)-GC-MS, increasing the sensitivity of the method. Other advantages of MASE, besides reducing the consumption of organic solvents, are the possibility of full automation, a drastically decreased extraction time and absence of emulsion in the extraction system.

MASE combined with LVI and GC-MS has been applied for the determination of triazines, PCBs, organochlorine and organophosphorus pesticides in complex aqueous matrices [3,4] Popp et al. determined PCBs in aqueous samples. A polypropylene membrane separated 15 mL water and 800 µL cyclohexane for 30 min in an agitator. Afterwards, 400 µL of the organic solvent was analyzed using large volume injection (LVI)-GC-MS. The obtained limits of detection varied between 2 and 10 ng/L [5].

3 Solid phase extraction

3.1 Principle

In solid phase extraction (SPE), a water sample is pumped through a solid phase, whereby the analytes are selectively adsorbed onto the surface of the solid phase. Afterwards, the adsorbent is washed to remove interfering matrix components. Finally, the analytes are eluted and further analyzed with GC-MS or LC-MS.

The principle or retention is analogous to high performance liquid chromatography (HPLC). SPE is suitable for pollutants with low, medium and high polarity depending on the solid phase selected. Most often, the cartridges are packed with a hydrophobic material such as octadecyl silica (C_{18}) or styrene-divinylbenzene co-polymer.

With SPE, large sample volumes can be handled using a relatively small amount of solid phase. This in turn requires only a small amount of organic solvent for the elution of the analytes, resulting in a significant sensitivity increase over classical techniques such as LLE. Furthermore, SPE cartridges have a low-cost which allows single use and they are commercially available in different formats with a diversity of solid phases.

Although SPE requires small volumes of organic solvent, it is often tedious and time-consuming. The manual version of SPE for concentrating samples with a large volume can take up to 8-10 h. It may present some disadvantages, e.g. breakthrough for large sample volumes. The extraction of analogues with different polarities is difficult and modified silica's are generally not resistant to pH extremes. Moreover, selectivity during analyte trapping is generally low due to the hydrophobic interaction mechanism. This can be overcome by using more selective types of adsorbent like molecularly imprinted polymers or immunosorbents.

After SPE extraction, the eluent can be analyzed with GC-MS or LC-MS. When GC-MS is applied, the extract is often evaporated to dryness under a gentle stream of nitrogen and reconstituted in a smaller volume. When analytes with functionalities

which are unsuitable for GC-MS analysis have to be determined, derivatization is carried out prior to analysis. When LC-MS is used, the extract can be directly analyzed.

A drawback of off-line SPE-LC-MS procedures is that they can be time consuming and cumbersome to perform, often requiring many steps before reaching a concentrated extract of which only a small portion is actually analyzed [6]. This can be overcome by performing on-line SPE, since this is performed faster and sample time is reduced. Thus, the sample throughput is increased. Other important advantages of on-line coupling are decreased risk of contamination of the sample extract, elimination of analyte losses by evaporation or by degradation during sample pre-concentration and improved precision and accuracy. Furthermore, higher sensitivity is achieved due to transfer and analysis of the totality of the extracted species to the analytical system. The most commonly used approach for on-line SPE is "column switching". The general set-up is given in **Figure I.12**.

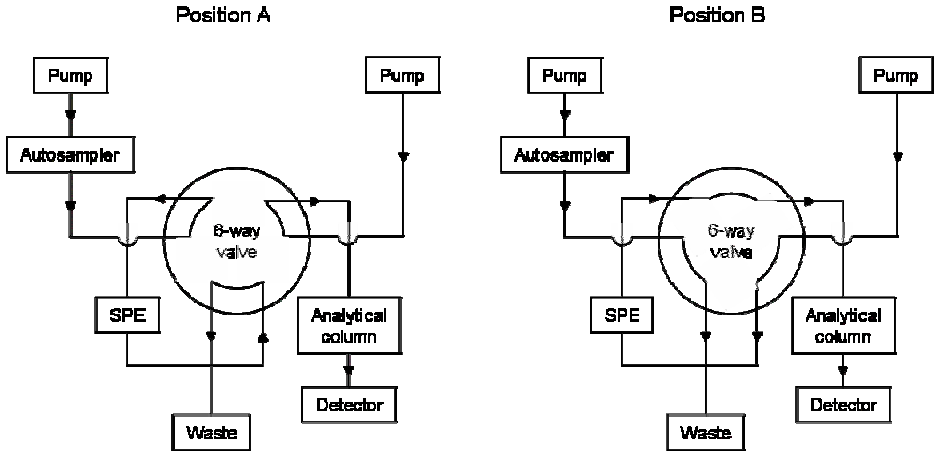

Figure I.12: General set-up for on-line SPE.

Column switching involves the implementation of a SPE cartridge within the injection loop of a six-port rotary valve. A sample is loaded on the pre-column when the valve is in position A. Afterwards the valve is switched to position B. The extracted compounds are now removed from the adsorbent by the LC mobile phase and introduced in the analytical column. Simultaneously with the analytical

separation, an exchange or re-conditioning of the cartridge takes place. Although, changing the cartridge after each analysis is very expensive, the reuse of the cartridge causes some problems, such as a progressive deterioration of the cartridge material leading to a change in selectivity and capacity. Another disadvantage is the risk of cross-contamination when complex or highly polluted samples are analyzed. Numerous applications of on-line SPE are available in the literature, but they will not be discussed here [7].

In the next paragraphs, the most common solid phases for SPE are summarized and their use for the analysis of EDCs is illustrated.

3.2 Reversed phase SPE

The most widely used mechanism for SPE is hydrophobic interaction. Common SPE sorbents reported in the literature for the trace enrichment of contaminants from various matrices are alkyl-bonded silicas (C_{18} silica), copolymer sorbents such as cross-linked polystyrene-divinylbenzene and hydrophilic-lipophilic balanced polymers.

Numerous applications of these sorbents for the determination of EDCs in environmental samples are available in literature. One example for each type of sorbent will be given.

The first type of sorbent, C_{18} silica, has been used by Barcelo et al. for the determination of natural and synthetic hormones in environmental matrices. An SPE cartridge containing 500 mg Lichrolut RP-18 cartridge was loaded with 1000 mL water sample. The analytes were desorbed using acetonitrile. Finally, the extract was analysed using LC-DAD-MS. The limits of detection reached with this method were in the range 1-20 ng/L [7].

The second type of solid phase is based on a copolymer of polystyrene-divinylbenzene. Alkylphenols and bisphenol A were already determined using this type of cartridge by Hagenmeier and coworkers. The SPE cartridge contained 200 mg polystyrene copolymer ENV+. A water sample of 1000 mL (drinking water or surface water) was first adjusted to pH 2. Then 5 mL MeOH and 5 g NaCl were added.

Elution of the cartridge was carried out using acetone. Afterwards the extract was evaporated to 50 µL under a gentle stream of nitrogen. The derivatizing agent phenyltrimethylammoniumhydroxide was added. Finally, the derivatized compounds were analyzed with GC-MS. The achieved limits of detection were lower then 0.05 ng/L [8].

A very popular SPE cartridge for the determination of EDCs is Oasis HLB®. This SPE material belongs to the third type of reversed phase sorbents since it is hydrophilic-lipophilic balanced [9]. Oasis HLB® is a co-polymer of the hydrophilic N-vinylpyrrolidone and the liphophilic divinylbenze. The combination of these two monomers allows the extraction of a wide variety of compounds. It has been successfully applied for the extraction of EDCs in aqueous samples by Wilding et al.. An Oasis HLB® cartridge of 200 mg was used for the analysis of natural and synthetic hormones, alkylphenols and bisphenol A. A sample volume of 500 mL was taken to load the cartridge. Elution was carried out with ethyl acetate and afterwards, the extract was evaporated to dryness under a gentle stream of nitrogen. Derivatization was performed with 50 µL N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% of trimethylchlorosilane at 60°C for 30 min. Finally, the extract was analyzed with GC-MS. This procedure was able to determine natural and synthetic hormones, alkylphenols and bisphenol A with a limit of detection between 0.3 and 5.3 ng/L [10].

All the reversed phase SPE materials lack selectivity. Co-extraction of analytes and matrix interferences generally occurs. Together with the target analytes, many matrix constituents can also be enriched and disturb the chromatographic separation and detection. Additional clean-up procedures are required, but the sample pre-treatment involves then several steps and consequently the risk of loss or contamination increases and the reliability of the results decrease. Furthermore, most polar compounds are difficult to enrich and often co-elute without retention with the interfering compounds of the polar matrix.

These drawbacks can be solved using selective tailor-made adsorbents like immobilized receptors or antibodies, molecularly imprinted synthetic polymers, and restricted access material.

3.3 Immuno-affinity extraction

Immuno-affinity extraction (IAE) is based on the highly selective antibody-antigen of receptor-hormone interaction and not on hydrophobic interactions. A specific biomolecule is immobilized on a suitable solid support producing a very selective immunosorbent [11]. The principle is demonstrated in **Figure I.13**

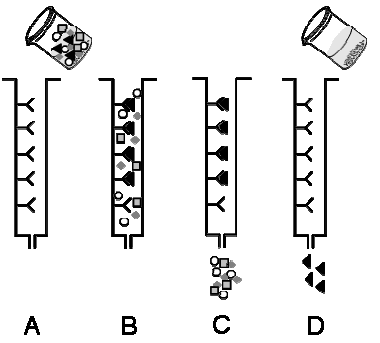

Figure I.13: Principle of immunoassay: Antibody (⊣) is bound on the solid phase and an aqueous solution is sent over the cartride (A). Both the antigen (◀) and matrix compounds (○■) are retained (B). The matrix is washed away (C) and finally the antigens are eluted (D).

The design of the antibodies is the key parameter that defines the potential of the immunosorbent. Two types of antibodies can be distinguished. Polyclonal antibodies are the first category. These include different antibodies that are able to recognise the same antigen. The other category contains the monoclonal antibodies. These are homogeneous, i.e. only one antibody recognizes one antigen. If antibodies are to be generated against small molecules like EDCs, they first have to be conjugated with a large protein like the bovine serum albumin to render them immunogenic [12]. The immunoresponse to this so-called hapten yields different antibodies. When extracted from the serum, these polyclonal antibodies can be used directly or first be purified to get only one type of monoclonal antibodies. The latter are much more selective and have less cross-reactivity then the polyclonal antibodies. Both polyclonal and monoclonal antibodies have been selected for immobilization. Monoclonal antibodies are frequently used because the results are more reproducible [13]. Polyclonal antibodies are more heterogeneous, leading to a high amount of cross-reactivity,

which is exploited in extraction because then all compounds within a given class can be enriched [14].

Instead of immobilizing the whole antibody on the solid phase, only the antigen binding domain could be attached, consequently increasing the number of binding sites on the solid phase without a negative effect on the steric hindrance. An augmentation in binding sites leads to an improvement of the capacity and thus also to an increase in breakthrough volume.

Although this type of sample preparation is very selective, even for aqueous samples, it still suffers from some drawbacks. For instance, when new analytes need to be determined, the antibodies have to be prepared and this can be very expensive and time-consuming. Besides, when a different immunization reaction is used, different antibodies can be produced. Furthermore, the antibodies are unstable in organic solvents, pH extremes and at higher temperatures. In addition, non-selective interactions with the solid phase may occur.

The use of antibodies for the determination of EDCs in aqueous samples has been described by Rhemrev-Boom et al.. Immunosorbents containing the antibody of 17-β-estradiol were prepared and their extraction efficiency towards 17-β-estradiol was evaluated. Loading of the cartridge was carried out using a 10 mM phosphate buffer (pH 7). Afterwards, the 17-β-estradiol was eluted with 80% MeOH. The cartridge was coupled on-line to LC-UV. Using this method, limits of detection of 2 µg/L were obtained [15].

Rhemrev-Boom et al. also immobilized the human estrogen receptor. In that way, analytes of interest could be selectively isolated from the matrix, by means of their biological activity. Compounds with xeno-estrogenic activity are captured by this affinity SPE cartridge. Loading of the cartridge was performed using 10 mM phosphate buffer, followed by a washing step with water. The analytes are then eluted with 25% v/v potassium thiocyanate buffer, 50% water and 25% methanol. The extract was analysed by LC-UV. The cartridge was able to quantitatively trap 17-β-estradiol. In a second set of experiments, the extraction of different phthalates was also investigated. It was found that only 6% of butylbenzylphthalate, which has only a

slight xeno-estrogenic activity, was captured. While DEHP, that is known to exhibit xeno-estrogenic activity, was captured quantitatively [15].

3.4 Molecularly imprinted polymers (MIP)

Molecular imprinting, which is becoming increasingly popular in recent years, is a technology where recognition sites are created by synthesizing highly cross-linked resins in the presence of a given molecule which act as a template. After the synthesis, the resin is thoroughly washed to remove the template and the resulting cavities are complementary in size and shape to the target molecule. It is claimed that these recognition sites mimic the binding sites of antibodies and receptors [16].

The most common approach to the MIP synthesis is non-covalent imprinting, which relies upon self-assembly of the template and a complementary functionalized monomer prior to the polymerisation. Thus, the template remains associated with the growing polymer during the synthesis and the addition of a large portion of cross-linking monomer allows the formation of complementary sites that remain stable after template removal [17]. The general principle of the synthesis of MIPs is shown in **Figure I.14**.

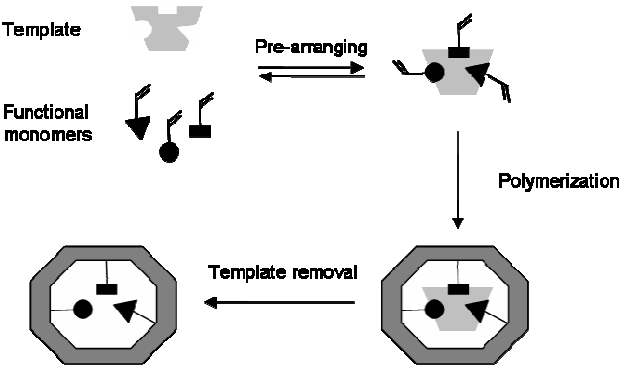

Figure I.14: General principle of the synthesis of MIPs.

The monomer is chosen in order to develop strong non-covalent interactions with the template. Widely used monomer and cross-linker are methacrylic acid and ethyleneglycol dimethacrylate, respectively. The appropriate solvent of such a polymerisation is generally an aprotic and non-polar solvent, because then the main

interactions between the template and the molecular imprint are hydrogen bonds and dipole-dipole interactions. The removal of the template molecule is the crucial step in the synthesis of MIPs. It is impossible to remove all the template molecules. A small amount always remains behind in the polymer despite the best attempts to extract it. Consequently, leaching of the template during subsequent enrichment of a sample can result in a positive error that can not be controlled. The easiest solution to this problem is using a template that is not the analyte of interest, but a suitable structural analogue. It has to be taken into account, that a small decrease in selectivity will occur when the analyte of interest is not used as template. The use of a structural analogue as template is also recommended when the target compound is toxic or very rare. Hosoya et al. prepared MIPs for the determination of bisphenol A, using 4-t-butylphenol as template molecule in order to avoid leaching of the target compound [18].

Notwithstanding the present hype, the use of MIPs for the analysis of aqueous samples presents some major problems. When the MIPs are prepared using non-covalent imprinting techniques, the dominant forces for the self-assembling of the monomers around the template are considered to be hydrogen bonding interactions. When aqueous samples are loaded on the MIP SPE cartridge, these hydrogen bonds are disturbed, while hydrophobic interactions are enhanced, leading to non-selective extraction of the analytes. Consequently, the usage of MIPs for the analysis of aqueous samples is limited. A few proposals to overcome this major drawback have been presented.

A first solution is to include a washing step with an organic solvent after loading of the aqueous sample. The non-selectively bound matrix components will be removed in this washing step and the analyte of interest that is retained on the MIP will switch from non-selective to selective binding. This has been applied by Moreno-Bundi et al.. They developed a MIP SPE cartridge for the determination of bisphenol A in aqueous samples. After loading of the aqueous sample, a washing step with acetonitrile was performed, in order to favour the specific interactions between the MIP and bisphenol A [19].

Another promising strategy is the combination of molecular imprinting with hydrogels. Hydrogels are cross-linked three dimensional hydrophilic polymer networks that swell when brought into contact with water. Because of their significant water content, hydrogels provide a degree of flexibility. Therefore, the challenge of this approach is the creation of effective imprinting structures providing sufficient rigidity/integrity of the binding pocket. Hiratani et al. reported that molecular imprinted hydrogels can be prepared by dissolving small amounts of methacrylic acid and ethylene glycol dimethacrylate in hydroethylmethacylate or N,N'-diethylacrylamide, that are the materials to prepare soft contact lenses. The resulting soft matrix showed higher affinity to timolol than the corresponding non-imprinted systems [20].

Adding 0.1% Triton 100 surfactant to the aqueous samples is described as another solution to limit non-specific interaction. This approach has been used by Meng et al. for the determination of 17-β-estradiol [21].

Finally, by modifying the surface of the MIPs with polar monomers such as 3-sulfopropyl methacrylic acid, any interference present in environmental water samples could be removed, resulting in a significant increase in sensitivity and more reliable results. Hosoya et al. used this procedure for the determination of bisphenol A in river water [22].

In conclusion, molecular imprinted polymers combine highly selective molecular recognition with typical properties of polymers such as high thermal, chemical and stress tolerance, and extremely long shelf-life, without any need for special storage conditions. In addition, MIPs are fast and easily prepared in a reproducible fashion when a suitable template is available. The low costs of the materials make single use of the cartridge possible. Although these advantages make enrichment using MIP a very promising technique, there are a lot of important drawbacks. For instance, when new analytes have to be determined, new MIPs have to be developed. They are able to extract a single analyte and sometimes its structural analogues, but they can not be used in a multi-residue method. Furthermore, leaching of the template can lead to positive errors.

Intensive research on the potential of MIPs for selective enrichment was performed in our research group for the determination of triazines in aqueous samples. To be honest, results were rather disappointing due to lack of selectivity [23].

3.5 Restricted access material (RAM)

Restricted access materials are bifunctional sorbents tailored for the fractionation (clean-up) of samples into macromolecular matrix components and low molecular-weight target analytes. Macromolecules are excluded by a physical barrier due to the pore diameter or by a chemical diffusion barrier created by a protein or a polymer network at the outer surface of the particle. Low molecular-weight analytes, able to access active adsorption centres at the inner pore surface, are retained by a reversed phase, affinity or ion pair mechanism depending on the type of adsorption centre [24].

RAMs have been successfully applied for direct extraction and enrichment of hydrophobic low molecular-weight analytes from biological fluids carrying a high load of proteins (like plasma, blood, urine, etc) and from food samples (milk, etc.) [25]. Applications where RAM was used for the clean-up of a sediment extract can also be found. For example, Petrovic et al. used RAM based SPE for the analysis of alkylphenolic compounds and steroid sex hormones in sediment. First, the sediment sample was subjected to pressurized liquid extraction. Afterwards, a clean-up of the obtained extract was performed using a LiChrospher ADS RAM cartridge from Merck. A scheme of the particles in this cartridge is given in **Figure I.15**

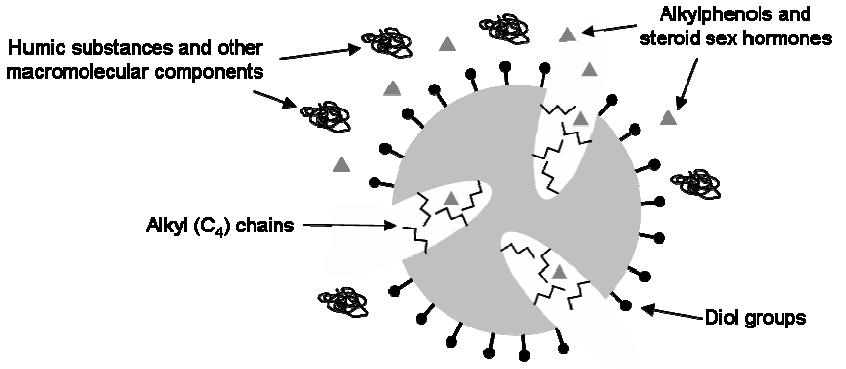

Figure I.15: Schematic overview of a particle of the RAM material of LiChrospher ADS RAM.

When the sediment extract is applied on the cartridge, first humic substances and other macromolecular components are eliminated by a size-exclusion mechanism. The analytes of interest, i.e. alkylphenols and steroid sex hormones, are retained by the alkyl C_4 chains inside the pores. The RAM extraction cartridge was on-line coupled to LC, for further analysis of the extracted analytes.

In conclusion, RAM is mostly interesting for the analysis of biological samples or sediment extracts, since these contain a large amount of macromolecular matrix components.

4 Sorptive extraction

In the present era of "green chemistry", modern approaches for sample preparation are in the direction of solventless extraction methods. Sorptive materials are ideal for this purpose. The principle of sorptive extraction will be explained, together with the two most important sample preparation techniques that utilizes this principle, namely solid phase micro-extraction (SPME) and stir bar sorptive extraction (SBSE).

4.1 Principle of sorptive extraction

Sorptive materials (or sorbents) are a group of polymeric materials with a glass transition temperature (T_g) below the temperature at which the material is used. Although, initially, this might seem a trivial requirement, the consequences are enormous. At temperatures above their Tg polymeric materials no longer behave as solid materials but assume a gum-like, or even a liquid-like, state with properties, e.g. diffusion and distribution constants, similar to those of organic solvents. **Figure I.16** shows the different transition states of a polymer by plotting the modulus of elasticity E against the temperature. The modulus of elasticity represents the degree of elasticity or the degree of 'stifness' of the polymer.

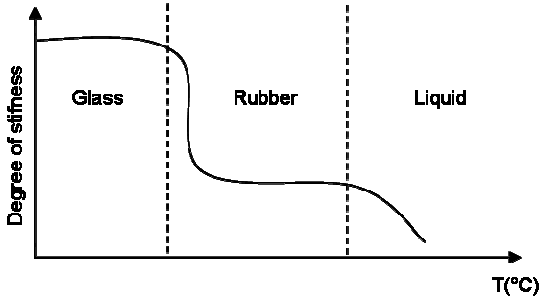

Figure I.16: Different states of a polymer expressed as modulus of elasticity in function of the temperature.

Three major states of the polymer can be distinguished. The first and the most "stiff" is the glass state. By increasing the temperature, the polymer segments become more and more mobile until the glass transition temperature is reached. From that

point on, the polymer is in the rubber state. When the temperature is further increased, the polymer becomes fluid. This occurs at the melting temperature T_f.

It is essential to realize that when sorbents are used, pre-concentration of analytes occurs by sorption of the analytes into the polymeric phase instead of adsorption on to a solid adsorbent surface. Consequently, the extraction of the analytes is not influenced by the presence of other matrix components.

The most commonly used sorbent is polydimethylsiloxane (PDMS) (T_g = -125°C) This phase is well known as a stationary phase in gas chromatography. It is so popular because PDMS is inert, can be used in a broad temperature range, its degradation products are very well known and easily identified by mass spectrometry and the enrichment of most analytes can be predicted. Another sorptive material is poly(butyl)acrylate, (T_g = -54°C). This is used for the extraction of more polar analytes, since their affinity towards PDMS is limited. Thermal stability of this sorptive material is less compared to PDMS leading to a higher amount of degradation products.

Since sorptive extraction is an equilibrium technique, the distribution of the analytes between the aqueous and the silicone phase is controlled by a partitioning coeffient $K_{pdms/w}$. Studies have correlated this partitioning coefficient with the octanol-water distribution coefficients $K_{o/w}$. It was found that over a specific polarity range $K_{o/w}$ and $K_{pdms/w}$ data correlate very well, especially for low-molecular weight analytes. However, for high molecular weight and very apolar solutes, e.g. polyaromatic hydrocarbons and polychlorinated biphenyl, the correlation between $K_{o/w}$ and $K_{pdms/w}$ seems no longer valid. For PCBs it was found by Yang et al. that with decreasing polarity (thus increasing $K_{o/w}$), the measured $K_{pdms/w}$ decreased [26]. The authors explained their data claiming that the PCBs were adsorbed onto the PDMS surface, rather than portioning into the bulk of PDMS. Mayer et al. were able to negate this statement. The partitioning of large apolar compounds such as PCBs and PAHs into the bulk of the PDMS was proven with fluorescence microscopy [27]. To find an explanation for the contradictory results obtained by Yang et al. further experiments were carried out by Baltussen et al. [28]. They analysed not only the SPME fiber but also the (Teflon coated) stir bar. The SPME recoveries were similar

to those reported by Yang, with recoveries of the most apolar PCB being lower than for the more polar (less chlorinated) compounds. This effect was, however, strongly counteracted by the discovery of substantially higher amounts of PCB on the stir bar. This clearly illustrated that the discrepancy between $K_{o/w}$ and $K_{pdms/w}$ values for very apolar and high molecular weight solutes is caused by the adsorption of the solutes onto the adsorbent surfaces present during the extraction (e.g. stir bar, glass vial, Teflon septum).

The octanol-water distribution coefficient $K_{o/w}$, although not fully correct, can be used to give a good indication if and how well a given solute can be extracted by PDMS. This can be expressed as the theoretical recovery η, that is defined as the ratio of the extracted amount of solute (m_{PDMS}) over the original amount of solute in the water ($m_0 = m_{PDMS} + m_w$), as described in the next equation:

$$\eta = \frac{m_{pdms}}{m_w + m_{pdms}} = \frac{1}{1 + \beta / K_{o/w}}$$

From this equation it can be seen that the theoretical recovery η can be calculated using the phase ratio β (with $\beta = V_w/V_{pdms}$) and the octanol-water distribution coefficient. The latter one can be calculated using the SRC-KOWWIN software package (Syracuse Research, Syracuse, NY, USA) according to a fragment constant estimation methodology [29].

From the equation of the extraction efficiency, it is clear that the extraction efficiency will decrease with increasing polarity of the analyte. Besides the $K_{pdms/w}$ factor, the phase ratio is also important. The higher the PDMS amount, the lower β and the higher the extraction efficiency will be. It should be noted that the theoretical recovery can only be achieved when equilibrium sampling is applied.

In **Figure I.17** the influence of $K_{o/w}$ and β on the extraction efficiency are demonstrated. A typical experiment utilizes 10 mL water. As will be further illustrated, when SPME is used, the volume of PDMS is approximately 0.5 µL, while in SBSE 25 µL is the most common PDMS amount. Equilibrium sampling is assumed.

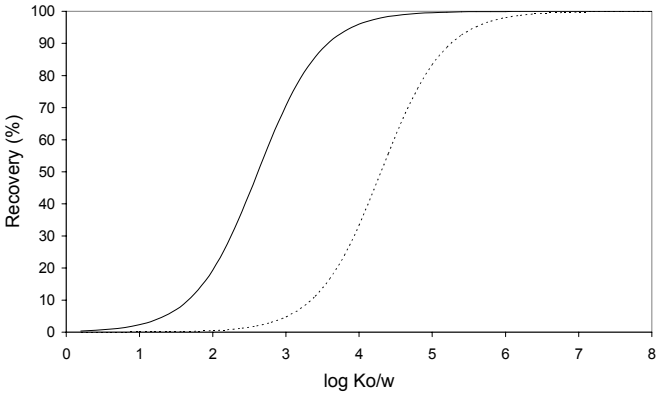

Figure I.17: Theoretical recovery (%) in function of log $K_{o/w}$ of the solutes for the analysis of a 10 mL water sample using SPME (100 µm fiber, 0.5 µL PDMS,----) and SBSE (1 cm x 0.5 mm d_f, 25 µL PDMS, ——). Equilibrium sampling is assumed.

From this figure, it is clear that quantitative extraction (100%) for SBSE is reached at much lower log $K_{o/w}$ values compared to SPME.

In conclusion, advantages of sorptive extraction include ease of use, high sensitivity, high reproducibility, no previous sample preparation is required, solvent-free and only small sample volumes are necessary.

4.2 Solid phase micro-extraction (SPME)

Solid phase micro-extraction (SPME) was introduced by Arthur and Pawliszyn in 1990 [30,31]. It employs a fused silica fibre with an outer diameter of typically 150 µm which is coated with an (ad)sorbent layer with a thickness of 5 to 100 µm. Consequently, the maximum volume of PDMS on the fiber is 0.5 µL. The small size of the SPME fiber and its cylindrical shape enable it to fit inside the needle of a syringe-like device. The set-up of this syringe is shown in **Figure I.18**.

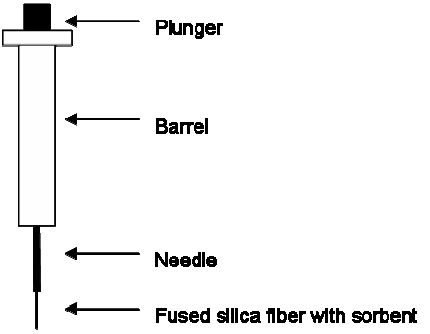

Figure I.18: Set-up of a SPME syringe.

The fused-silica fiber is located inside the barrel of the syringe for protection when not in use. The needle of the syringe is then used to pierce the septum of the vial for sampling. At this point, the fiber is exposed to the analytes by pressing down the plunger for a pre-determined time. Afterwards, the fiber is withdrawn back into its protective syringe barrel and withdrawn from the sample vial. Finally, the SPME device is inserted into the hot injector of the gas chromatograph and the analytes are desorbed from the fiber prior to GC separation and detection.

The simplicity and performance of SPME created a lot of interest in this technique. In addition, a normal GC inlet can be used for desorption of the fiber and automation of the procedure is possible. The only major disadvantage is the limited amount of sorbent attached on the fiber. For a typical 100 µm polydimethylsiloxane fiber, which is the most widely used fiber, the volume of extraction phase is approximately 0.5 µL. Consequently, only analytes with log $K_{o/w}$ > 6 can be quantitatively extracted (**Figure I.17**). This is a major drawback of SPME.

To improve the capacity of SPME fibers, several 'new' SPME coatings have been introduced. These include materials such as co-polymers of PDMS with divinylbenzene (PDMS-DVB), and Carbowax (PDMS-WAX) and physical mixtures of PDMS with adsorbents such as Carboxen. Although these materials do indeed, significantly increase trapping capacity for some solutes, the true sorption mechanism is lost. Carbowax for example is used below its glass transition temperature (70°C) and Carboxen is an inorganic adsorbent. Consequently, the extraction will occur by adsorption rather then sorption and the matrix compounds (humic acids, proteins, etc.)

will thus compete with the target analytes for available adsorbent sites, complicating reliable quantification in SPME.

SPME has intensively been used for the determination of EDCs in aqueous samples. For example, Braun et al. compared 100 µm PDMS, 65 µm PDMS-DVB and 85 µm polyacrylate fibers for the analysis of nonylphenols, bisphenol A and ethinyl estradiol in waste water. Salt was added to decrease the solubility of the analytes in water. Furthermore, the water sample was acidified to pH 2 in order to protonate the solutes, thus increasing the extraction efficiency. It was concluded that polyacrylate was the most suitable sorbent [32]. To improve the GC analysis of these types of compounds, a derivatization step can be carried out. This will also have a beneficial effect on the sensitivity of the method. This was demonstrated by Yang et al.. SPME with a 85 µm polyacrylate fiber was used for the analysis of alkylphenols and steroid hormones in environmental and biological samples. Following SPME, derivatization was performed by exposing the fiber to the vapours of BSTFA. Finally, GC-MS analysis was carried out by placing the SPME fiber in the GC inlet [33].

Up till now SPME sampling directly in the aqueous phase has been discussed. An alternative to this type of sampling is SPME extraction in the headspace of the sample. This was described theoretically by Zhang and Pawliszyn [34] and by Ai [35]. In headspace SPME volatile analytes are extracted and concentrated on the SPME coating. This can have several advantages over direct SPME extraction in the liquid phase. For instance, equilibration times can be reduced substantially, because diffusion coefficients are higher in the gas than the liquid phase. Furthermore, the analysis of samples containing high-molecular weight or particulate material can be carried out with greater accuracy using headspace SPME. Fiber lifetime is also extended, because these unwanted compounds do not come into contact with the fiber.

Headspace-SPME is not only a successful approach to the analysis of gaseous and liquid samples but can also be used for solid samples or even for the direct analysis of air samples.

An application of headspace SPME for the analysis of EDCs is the determination of organotin compounds in water samples as described by Devos et al. [36]. A

complete automated procedure using headspace SPME in combination with derivatization and GC-MS was developed. The extraction was carried out using a 100 µm PDMS fiber. After sampling, the SPME fiber was analyzed by GC-MS. During the headspace extraction, in-situ derivatization of the aqueous sample was performed with sodium tetraethylborate. Hence, the extraction efficiency was improved as was the chromatographic performance for these compounds [37].

4.3 Stir bar sorptive extraction (SBSE)

Stir bar sorptive extraction was developed by Baltussen et al. to improve the extraction efficiency compared to SPME [38]. This was achieved by applying a higher volume of PDMS (typically 20-200 µL) for the extraction. A stir bar of 1 to 2 cm long is coated with a 0.5 or 1 mm layer of PDMS. The stir bar is shown in **Figure I.19**.

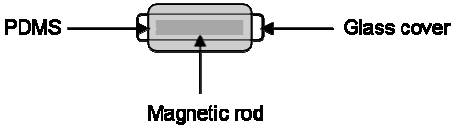

Figure I.19: Stir bar coated with PDMS for SBSE.

As a consequence of the higher amount of PDMS, the phase ratio β was decreased and thus, for the same log $K_{o/w}$, the theoretical extraction efficiency was increased. This is illustrated in **Figure I.17**, where the recoveries of SBSE and SPME are shown in function of the log $K_{o/w}$ values. From this figure, it can be concluded that SBSE is able to quantitatively extract analytes with lower log $K_{o/w}$ compared to SPME. Consequently, detection limits at the sub-ng/L level can be reached by SBSE.

Stir bar sorptive extraction is performed by placing a suitable amount of sample in a headspace vial or other container. The stir bar is added and the sample is stirred for a pre-determined time between (typically between 30 and 240 min). Afterwards, the stir bar is removed, rinsed slightly with distilled water to remove adsorbed sugars, proteins, or other sample components and finally dipped on a clean paper tissue to remove water droplets. It should be noted that rinsing does not cause loss of analytes

because the sorbed solutes are localized inside the PDMS phase. Finally, the analytes are recovered by either liquid or thermal desorption.

In the first case, a back-extraction of the stir bar is performed with an organic solvent. This is normally used in combination with HPLC [39] or large volume injection GC [40] in order to obtain the highest possible sensitivity. The combination of liquid desorption with HPLC was utilized by Kawaguchi et al. for the determination of 4-tert-octylphenol and nonylphenol in animal feed samples. Prior to SBSE extraction, the animal feed was sonicated with methanol. Then, 5 mL of the supernatant was diluted with water to 20 mL. In this solution SBSE extraction was carried out, followed by ultrasonic liquid desorption with 200 µL acetonitrile. Finally, the extract was analysed using HPLC [41]. The combination of liquid desorption with large volume injection GC-MS has been reported by Serôdio et al. for the determination of a large group of endocrine disruptors. Here, the liquid desorption was carried out with 100 µL acetonitrile. Then, the extract was evaporated and re-dissolved in 80 µL ethyl acetate of which 20 µL was analysed by LVI-GC-MS. It should be noted that complete transfer of the extracted analytes to the analytical column is impossible using liquid desorption, so in that case thermal desorption is preferred.

Thermal desorption is the second desorption approach. In SPME, thermal desorption is perfomed in the inlet (typically split/splitless inlet) of a gas chromatograph. This approach cannot be used for SBSE since more sorptive material is used, leading to longer desorption times (10 min) and the need of higher desorption flows (100 mL/min). Therefore, thermal desorption of the stir bars is carried out using a commercially available thermal desorption unit (Gerstel GmbH,Müllheim a/d Ruhr, Germany) as is shown in **Figure I.20**.

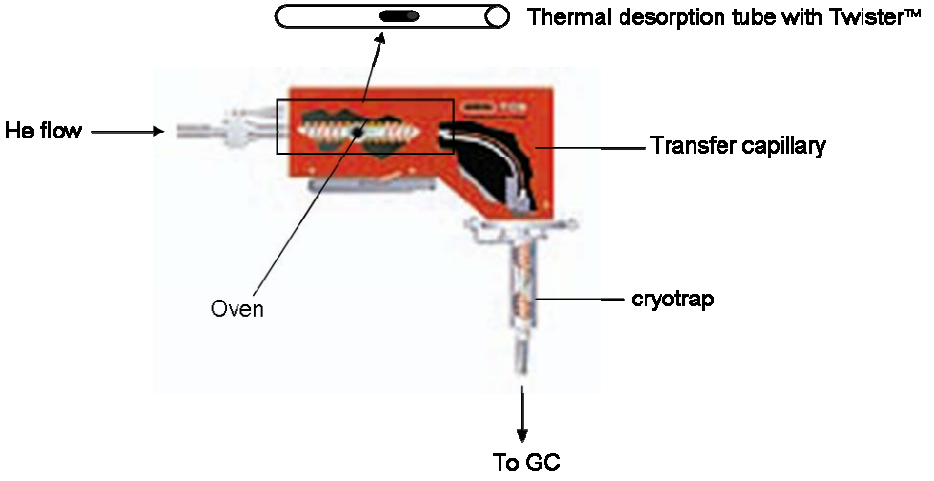

Figure I.20: Set-up of the dedicated thermal desorption unit.

Thermal desorption is carried out by introducing the stir bar in a glass tube which is then placed in the thermal desorption unit. The desorption temperature can be programmed like the temperature program of a GC oven. Since the time of desorption is typically 10 min, focussing of the analytes before entering the analytical column is necessary. In this set-up, the thermally desorbed analytes are trapped in a cryotrap which is actually a programmed-temperature vaporizing (PTV) injector, set at temperatures as low as −150 °C using liquid nitrogen cooling. Most of the applications with SBSE, use thermal desorption. For example, the determination of nonylphenol and octylphenol in water was presented by Kawaguchi et al. After the SBSE extraction, thermal desorption was performed followed by GC-MS analysis [42].

While normally only one stir bar is desorbed, the simultaneous thermal desorption of five stir bars in the so-called "multi-shot" mode was presented by Kawaguchi et al. for the determination of estrogens in river water [43]. An enormous increase in sensitivity was observed using this approach.

After thermal or liquid desorption, the stir bars can be reused. Typically, the life-time of a single stir bar varies from 20 to more than 50 extractions, depending on the matrix.

When using SBSE, analytes with a wide variety in polarity can be extracted by adjusting the extraction conditions. For instance, the extraction of highly apolar solutes, such as PAHs and PCBs suffer from competitive glass adsorption as mentioned previously in Chapter II.4.1. This problem can be overcome by adding an organic modifier like methanol [44,45] or hyamine [45] (ionic tenside) to the aqueous solution. The addition will effect the distribution of the analytes between the PDMS and the water phase, but for those applications, the overall effect is higher recovery.

The extraction of polar compounds normally results in low recoveries since they are characterized by low log $K_{o/w}$ values. The extraction efficiency can be improved using *in-situ* derivatization reactions, since these reactions increase the log $K_{o/w}$ values of the solutes. For example, the log $K_{o/w}$ of 3.4-dichloroaniline is 2.37. After *in-situ* derivatization with ethyl chloroformate, 3.4-dichloroaniline ethyl carbamate is is formed with a log $K_{o/w}$ value of 3.53. Typical derivatization reactions that can be performed in aqueous media include acetylation of phenols using acetic acid anhydride [46], esterification of acids and acylation of amines using ethyl chloroformate [47], oximation of aldehydes and ketones using pentafluorobenzyl hydroxylamine [48] and ethylation of organotin compounds using tetraethylborate [36]. It is also possible to derivatize the analytes after extraction to improve chromatographic performance and/or detectability. For instance, in-tube silylation has been described by Kawaguchi et al. for the determination of alkylphenols in water samples. After SBSE extraction, the stir bar was placed in the desorption tube. In the back portion of the glass desorption tube a glass capillary filled with 0.5 µL BSTFA was placed. During the thermal desorption the derivatization is performed [49]. It should be noted that silylation cannot be performed in aqueous media, since the derivatized analytes are easily hydrolyzed. More detailed information about the possible *in-situ* derivatization reactions is given in Chapter III.3.3.1. Another solution for the limited recovery of polar compounds is the addition of salt, also referred to as the "salting out" principle. By adding salt to the aqueous solution, the solubility of the polar compounds will decrease, hence improving the extraction efficiency. Leon et al investigated the influence of salt on the SBSE recovery of pesticides [50].

In accordance to SPME, headspace sampling is also possible with SBSE. The stir bar can be placed above a liquid or solid sample and special devices to hold the stir bar are available. Headspace SBSE has already been applied by Bicchi et al for the analysis of the headspace of medicinal plants [51].

Stir bar sorptive extraction has been used for several applications, most of them in combination with thermal desorption. They have recently been reviewed by Kawaguchi et al. [52] and David et al. [53].

The only disadvantage of SBSE is that the only stir bars that are commercially available are those with PDMS. (Twister™, Gerstel GmbH, Müllheim a/d Ruhr, Germany). Recently, also other phases have been developed and evaluated. Liu et al. described the use of sol–gel technology to obtain thin (30 μm) layers of PDMS on stirring rods [54,55]. Hu et al. also used sol-gel technology. A new type of material based on PDMS was prepared, but β-cyclodextrin was incorporated in order to improve the extraction of polar analytes [56]. Lambert et al. coated restricted access material (RAM) on stir bars for the extraction of caffeine (log $K_{o/w} = -0.1$) and metabolites in biological fluids. The principle of the restricted access material is descibed in Chapter II.3.5. The RAM particles used for the stir bar coating are analogous to the ones shown in **Figure I.15**. The outer surface is covered with diol groups, while the inner surface contains C_{18} alkyl chains. The RAM-coated stir bars were used for the direct extraction of caffeine and its metabolites in biological fluids in combination with liquid desorption followed by liquid chromatography [57]. Bicchi et al. described the use of a dual phase stir bar both in SBSE (immersion) mode and in headspace (HSSE) mode. These new stir bars consisted of an outer PDMS coating and a carbon adsorbent material inside. Magnetic stirring is possible by two small magnets placed at the ends of the stir bar. A schematic diagram of the dual phase stir bar is given in **Figure I.21**.

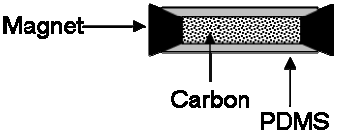

Figure I.21: Diagram of the dual phase stir bar as presented by Bicchi et al..

This dual phase device, whereby sorption is combined with adsorption (on the carbon material), showed increased recovery of very volatile compounds emitted from plant material and also higher recoveries are obtained for more polar solutes in water samples. These dual phase stir bars are analysed by thermal desorption followed by GC-MS [58]. Neng et al proposed polyurethane foams as coating for the stir bars. They were used for the analysis of atrazine in water samples in combination with liquid desorption and HPLC-UV or LVI-GC-MS [59]. Recently, Huang et al. demonstrated the use of different monolithic materials as coating for the stir bars. Different monomers and crosslinkers have been used for their preparation, leading to monoliths with different extraction capabilities. The first type was prepared with octyl methacrylate as monomer and ethylenedimethacrylate as crosslinker. This was succesfully used for the determination of the apolar PAHs in seawater samples. The extraction efficiency for polar analytes, on the other hand, was limited [60]. Therefore, a new monolithic material poly(methacrylic acid stearyl ester-ethylenedimethacrylate) was synthesized and used for the analysis of steroid sex hormones in urine samples [61]. As a variation on the latter, poly(4-vinylpyridine-ethylenedimethacrylate) was synthesized and applied for the determination of phenols in lake water [62]. The stir bars containing monolithic material were always combined with liquid desorption followed by LC-UV.

Alternative designs have also been used. For example, Montero et al. demonstrated the use of "PDMS rods" with dimensions up to 8 cm long and coated with 250 µL PDMS for the analysis of PCBs [63]. Another approach was presented by Popp et al.. A silicone tube of 1 cm was used for the extraction of triazines and PCBs in water samples. Afterwards, the silicone tube was desorbed in 200 µL cyclohexane and further analysis was carried out with large volume injection GC-MS [64].

In conclusion, when SBSE is used as sample preparation technique, detection limits at the sub-ng/L level can be reached. Furthermore, the extraction of polar solutes can be improved by using *in-situ* derivatization reactions. Finally, the search

for new materials to replace PDMS is ongoing, but so far a suitable and universally applicable substitute for PDMS has not been found.

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CHAPTER III
DEVELOPMENT OF A MULTI-RESIDUE METHOD FOR THE DETERMINATION OF EDCs IN AQUEOUS SAMPLES

In this chapter, stir bar sorptive extraction (SBSE) was first applied to the enrichment of pyrethroids in water samples to evaluate the different desorption techniques. Thermal desorption (TD) was performed in a classical split-splitless inlet equipped with a flip-top sealing system and in a dedicated thermal desorption unit. These two thermal desorption methods were compared to liquid desorption with ethyl acetate. Several parameters that influence extraction and desorption efficiency were evaluated. The performances of the methods were evaluated in terms of recovery, linearity, repeatability and limits of detection (LODs). Sensitivity was the highest for thermal desorption in a dedicated thermal desorption unit.

Therefore, this procedure was used for the development of a multi-residue method for EDCs in aqueous samples. Four different sample preparation procedures carried out in parallel on four aliquots of the same water sample are performed. Three derivatisation reactions specific to phenolic compounds, amines and acids, and organometallic compounds, respectively, were applied to three sample aliquots, while compounds with a log $K_{o/w}$ compatible with PDMS and not requiring derivatisation were sampled in the fourth aliquot. In-tube silylation was carried out with BSTFA. The resulting stir bars are introduced in the same thermal desorption tube, heat desorbed and analysed simultaneously by capillary GC-MS. The figures of merit of the method were evaluated with an EDC model mixture. The performance of the method is illustrated with the analysis of some real water samples.

1 Introduction

In the last decade, worldwide concern has been dedicated to the increasing distribution of endocrine disrupting chemicals (EDCs) in the environment. This anxiety is caused by the adverse effect of these pollutants on the hormone system of humans and wildlife as is illustrated in Chapter I.3. Therefore, highly sensitive methods are needed to evaluate potential risks. An overview of the techniques used for the analysis of EDCs in aqueous samples was given in Chapter II. All these methods include a pre-concentration step in order to determine these compounds at the very low concentrations at which they are present in environmental water samples. Whereas all these methods are single or selective residue methods to screen particular classes of compounds, the development of multi-residue methodologies to monitor as many compounds as possible in only one sample preparation procedure and chromatographic technique should have a great value for screening purposes. Since EDCs are chemically very heterogeneous, the search for an appropriate multi-residue method is a challenging and complex task.

As a consequence, the used sample preparation procedure should be able to enrich low concentrations of solutes with a large variety in polarity. Stir bar sorptive extraction is suited for this purpose as is described by David et al.[1]. Therefore, SBSE was used as sample preparation technique for the development of a multi-residue method in combination with GC-MS. First, desorption of the stir bars is optimized for the analysis of pyrethroids in water samples. Thermal desorption (TD) was performed in a classical split-splitless inlet equipped with a flip-top sealing system and in a dedicated thermal desorption unit. These two thermal desorption methods were compared to liquid desorption with ethyl acetate. Afterwards the most sensitive desorption technique was used for the multi-residue method for the determination of EDCs.

EDCs are a heterogeneous group of compounds that can be divided into five different sub-groups, based on their functionality. The first group contains phenolic EDCs. The amine-based EDCs belong to the second group. The third group includes the acidic EDCs. The fourth group consists of the organotin compounds. The last

group incorporates EDCs with log $K_{o/w}$ values > 5. Since the apolar PDMS phase is not highly effective to extract highly polar compounds, *in-situ* derivatization was carried out. Different *in-situ* derivatization procedures are evaluated and optimized for each group of EDCs. Afterwards, a method was searched that was able to analyse all the stir bars in one analysis. The method was developed on some representative EDCs for each group using labelled internal standards and then extended to a larger number of them and of pharmaceuticals as well. The performance was evaluated in terms of linearity, repeatability and limits of detection (LODs). At last, the method was applied to screen and quantify EDCs and some pharmaceutical residues in process waters and hospital effluent water.

2 Analysis of pyrethroids in water samples using SBSE-GC-MS: Evaluation of different desorption techniques*

2.1 Introduction

Pyrethrins and pyrethroids are powerful insecticides that, in the last decades, increasingly have replaced organochlorine pesticides due to their relatively low mammalian toxicity, selective insecticide activity and low environmental persistence. Pyrethroids are the synthetic derivatives of pyrethrins, the naturally toxic constituent of the flowers of the Chrysanthemum plants. Pyrethrins have been used as insecticides for more than a century, but because they have low photostability, the synthetic pyrethroids were developed. In an example of both pyrethroids as pyrethrins are given.

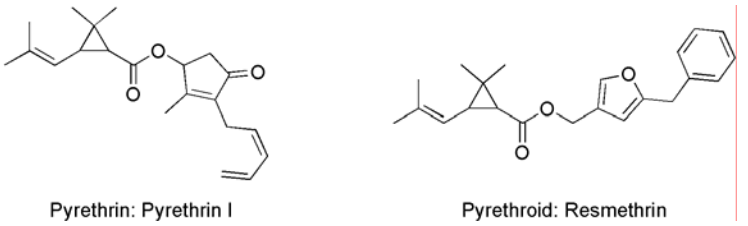

Figure III.1: Structures of a pyrethrin (Pyrethrin I) and a pyrethroid (Resmethrin).

Some commercial pesticide formulations containing pyrethroids are Arrivo (cypermethrin), Cyhalon (λ-cyhalothrin) and Ambush (permethrin) [2].

Although pyrethroids are thought to be safe for humans, they are highly toxic to fish even at very low concentrations (< 0.5 µg/L water) [3]. After exposure of humans to pyrethroids some reversible symptoms of poisoning and suppressive effects on the

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immune system have been reported [4]. The pyrethroids have been included in a list of suspected endocrine disrupting chemicals by an EU working group [5]. Consequently, these target compounds should be monitored at ultra-trace level, well below the maximum contaminant levels for pesticides (0.1 µg/L) mentioned in the EU drinking water quality directive [6].

Pyrethroids are analysed by different methods [7], including gas chromatography (GC) combined with electron capture detection (ECD) [8] or mass spectrometry (MS) [9-15] and liquid chromatography (LC) [16].

Pyrethroid analysis in water samples at trace levels requires a pre-concentration step. Pyrethrins and pyrethroids are apolar in nature as reflected by their high octanol-water partitioning coefficients (log $K_{o/w} > 5$) as shown in **Table III.1**. Common methods for the pre-concentration of pyrethroids from aqueous samples are liquid-liquid extraction (LLE) [12] with an organic solvent like hexane or methylene chloride and solid phase extraction (SPE) [8]. The solventless extraction techniques solid phase micro extraction (SPME) [17] and stir bar sorptive extraction (SBSE) [18] were successfully applied for the determination of pyrethroids. In the latter publication, SBSE was combined with liquid desorption and large volume injection GC-MS. Main advantage of SBSE [19] over SPME [20] is the much larger volume of the extracting phase (20 µL to 200 µL for SBSE versus 0.5 µL for SPME) which results in detection limits at the sub-ng/L level.

Disadvantage of SBSE over SPME, at least in the high sensitivity thermal desorption mode, is the need of a dedicated expensive thermal desorption unit. As alternatives, Bicchi et al. described thermal desorption directly in the GC liner [21,22] and Popp et al. developed liquid desorption by back extracting the stir bar with an organic solvent for LC analysis of polycyclic aromatic hydrocarbons [23]. Liquid desorption in combination with large volume injection GC was logically the next step in SBSE desorption [24].

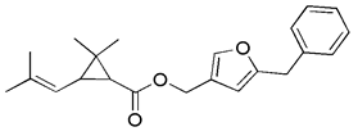
The aim of this study was to compare the different desorption modes i.e. thermal desorption in a dedicated desorber, in a classical split/splitless inlet equipped with a flip-top device [36] and by liquid desorption. GC-MS in SIM mode was used in all cases. Several parameters that influence extraction and desorption efficiency were

evaluated. The performances of the methods were evaluated in terms of recovery, linearity, repeatability and limits of detection (LODs).

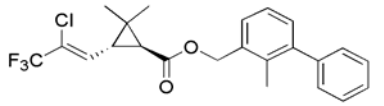
2.2 Experimental

2.2.1 Chemicals

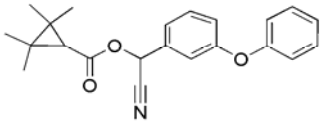
Neat certified pyrethroid standards were purchased from different sources. Resmethrin (mixture of cis- and trans isomers) (purity 94.3%), fenpropathrin (98.3%), λ-cyhalothrin (99.7%), acrinathrin (99.8%), deltamethrin (99.8%), fenvalerate (99.8%) and esfenvalerate (99.9%) were from Riedel de Haën (Seelze, Germany). Bifenthrin (99.5%) and cypermethrin (91.0%) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Cis-permethrin (99%) was supplied by Chem Service (West Chester, USA). Trans-permethrin (94%) was purchased from Alltech (Lokeren, Belgium)**.** The internal standard Trans-permethrin-d_6 (dimethyl-d_6) (97%) and chlorpyrifos (98.4%) used for retention time locking were purchased from Dr. Ehrenstorfer (Augsburg, Germany). The locked retention times are presented in **Table III.1**. The chemical structures of the pyrethroids are given in **Figure III.2**. Water, methanol, acetone, acetonitrile and ethyl acetate (all HPLC grade) were purchased from Sigma-Aldrich (Bornem, Belgium). Stock solutions of each individual compound were prepared in acetone at a concentration of 1 mg/mL. This solution was stored at 4°C and used to prepare the spiking solutions.



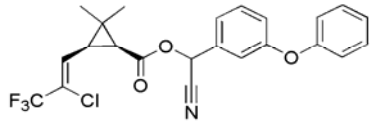
Resmethrin



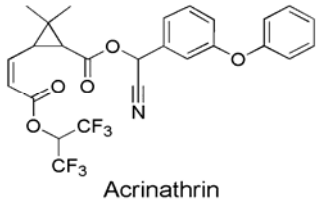
Bifenthrin



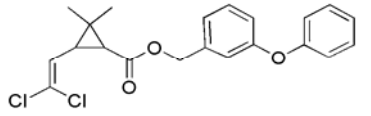
Fenpropathrin



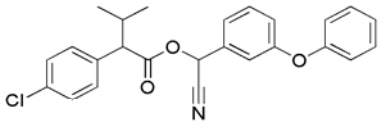
λ–Cyhalothrin



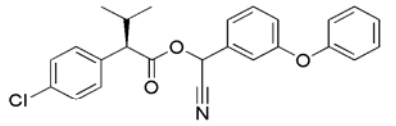
Acrinathrin



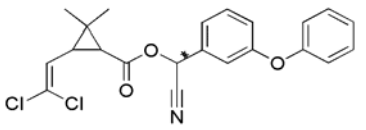
Permethrin



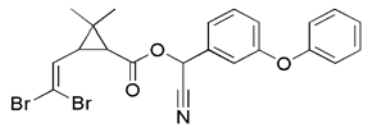
Fenvalerate



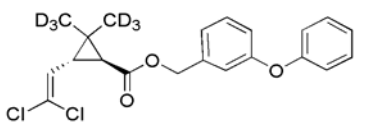
Esfenvalerate



Cypermethrin



Deltamethrin



Trans-permethrin-d_6
(dimethyl d_6)

Figure III.2 Structures of the pyrethroids

2.2.2 Sample preparation

Stir bars (10 mm x 0.5 mm containing 25 µL polydimethylsiloxane-PDMS coating) (Twisters) were obtained from Gerstel (Gerstel GmbH, Mülheim an der Ruhr, Germany). Method development was done using 10 mL water samples spiked with 10 µL of a 100 ng/mL pyrethroid standard solution and 10 µL of the trans-permethrin-d_6 internal standard solution at the same concentration. This corresponds to a concentration of 100 ng/L (ppt) in the water sample (or 1 ng per solute added to 10 mL sample). 0 to 3 mL MeOH were added to the water sample to minimize wall adsorption and then the stir bar was added. Extraction was performed at room temperature while stirring at 900 rpm (Variomag Multipoint 6/15, H+P Labortechnik, München, Germany) for an extraction time ranging from 30 to 360 min. Afterwards the stir bars were removed from the aqueous solution with tweezers, dried on a lint-free tissue and thermal or liquid desorption was performed.

2.2.3 Instrumental

Analyses were carried out on an Agilent 6890 gas chromatograph – 5973 mass spectroscopic detector combination (Agilent Technologies, Little Falls, DE, USA) equipped with a split/splitless injector and a programmed temperature vaporization inlet (CIS-4, Gerstel GmbH, Mullheim, Germany). For thermal desorption in the conventional split/splitless (S/SL) inlet, the injector was equipped with a flip-top sealing system (Agilent). This flip-top device has a levered arm that attaches to any Agilent (6890/6850/5890) S/SL insert weldment and is locked to the injection port using an adapter ring screwed onto the inlet. By simply lifting the arm of the flip-top, the insert weldment is released from the injection port giving instant access to the liner. The process is simply reversed to re-seal the weldment to the port. For thermal desorption of a stir bar, the injection port was opened by lifting the arm of the flip-top. The stir bar was placed in a 4 mm ID single baffled liner (5181-3316, Agilent Technologies) and the arm of the flip-top was closed. During the entire procedure, the liner is not taken out of the injector. The liner was maintained at 300°C during the whole analytical sequence. The carrier gas helium was supplied in the constant

pressure mode and injection was performed in the splitless mode (flow 1.5 mL/min). After 5 min, the purge vent was switched on to 50 mL/min. After 6 min the gas saver flow, which was set to 20 mL/min, was switched on. After introduction of the stir bar in the liner, the GC analysis was started immediately.

This type of thermal desorption was compared to conventional stir bar thermal desorption using a TDS-2 unit (Gerstel GmbH, Müllheim, Germany) mounted on the GC via the CIS-4 inlet. Here the stir bar was placed into a glass tube of 187 mm L, 6 mm OD and 4 mm ID. Splitless thermal desorption was performed by programming the TDS from 35°C (1 min) to 300°C (5 min) at a rate of 60°C/min with a helium flow rate of 100 mL/min. The analytes were cryo-focussed in the CIS-4 inlet at -150°C using liquid nitrogen. Splitless injection was performed by ramping the CIS-4 from -150°C (0.10 min) to 300°C (5 min) at a rate of 10°C/s.

For liquid desorption the stir bars were placed in a vial equipped with small volume insert (Alltech, Lokeren, Belgium) and 150 µL of acetone, acetonitrile or ethyl acetate were added, hereby completely immersing the stir bar. Chlorinated solvents such as methylene chloride were not considered because they damage the PDMS phase [37]. The extraction was performed in an ultrasonic bath for 15 to 60 min at room temperature. Afterwards the stir bar was removed and 1 or 10 µL of the extract were injected in the CIS-4 injector operated in the solvent venting mode.

The analyses were performed on an HP-5MS fused silica capillary column (5% diphenyl, 95% dimethylsiloxane) of 30 m L, 0.25 mm ID and a phase thickness of 0.25 µm (Agilent Technologies, Folsom, USA). The oven was programmed from 70°C (2 min) to 150°C at 25°C/min, then to 200°C at 3°C/min and finally at 8°C/min to 280°C (10 min). The head pressure of the carrier gas was adjusted using the retention time locking (RTL) software (Agilent Technologies) so that chlorpyrifos was eluting at a constant retention time of 19.23 min. Detection was carried out in the selected ion monitoring (SIM) mode. The transfer line, ion source and quadrupole analyser temperatures were set at 280°C, 230°C and 150°C respectively, and a solvent delay of 4 min was used. Electron ionisation mass spectra were recorded at 70eV electron energy with an ionisation current of 34.6 µA. Two characteristic ions for each compound were selected namely a target ion for quantification and a qualifier

ion. The SIM groups are listed in **Table III.1**. The dwell time was 100 ms. Data acquisition, instrument control and data analysis were performed by ChemStation software (G1701CA, version C.00.00, Agilent Technologies).

Table III.1: Octanol-water partition coefficients, retention times and selected SIM ions for the pyrethroids studied.

Pyrethroids	Log $K_{o/w}$ [a]	Retention time (min)	SIM ions [b]	SIM group
Cis-Resmethrin	7.11	27.79	*123*/128	1
Trans-Resmethrin	7.11	27.97	*123*/128	1
Bifenthin	8.15	28.87	*181*/165	2
Fenpropathrin	5.62	29.00	*97*/181	2
λ-Cyhalothrin	6.85	30.40	*181*/197	3
Acrinathrin	6.73	30.73	*181*/93	3
Cis-Permethrin	6.18	31.40	183/*163* [c]	4
Trans-permthrin-dimethyl-d_6 (I.S.)		31.54	183/*169* [c]	4
Trans-permethrin	6.18	31.58	183/*163* [c]	4
Cypermethrin I	6.38	32.73	*181*/163	5
Cypermethrin II	6.38	32.89	*181*/163	5
Cypermethrin III	6.38	33.01	*181*/163	5
Cypermethrin IV	6.38	33.07	*181*/163	5
Fenvalerate	6.76	34.33	*125*/167	6
Esfenvalerate	6.76	34.75	*125*/167	6
Deltamethrin	6.18	35.92	*181*/253	7

[a] Octanol/water coefficients are obtained by the software program KOWWIN

[b] Target ions in *italic*

[c] The most abundant ion is actually 183 but this is the same for both trans-permethrin and trans-permethrin-dimethyl-d_6, therefore m/z 163 and 169, respectively, were selected as target ion

In order to clarify the data presented in the previous table, the following remark should be made. When the mass spectra of trans-permethrin and trans-permethtrin-d_6 are compared, the most abundant ion is for both compounds the same as is demonstrated in **Figure III.3**.

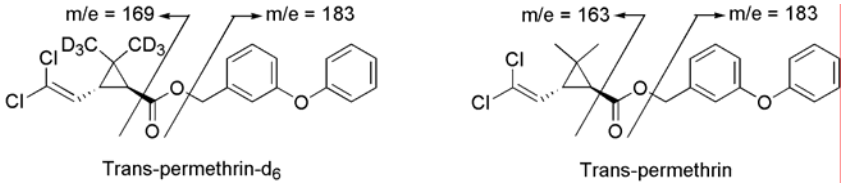


Figure III.3; Fragmentation of trans-permethrin and trans-permethrin-d_6 using electron impact.

Since trans-permethrin and trans-permethrin-d_6 are not completely separated (**Figure III.6**), a different ion should be chosen for the quantification. Consequently, 163 and 169 are used for trans-permethrin and trans-permethrin-d_6, respectively.

2.3 Results and discussion

2.3.1 Optimisation of the sample preparation

As is mentioned before, stir bar sorptive extraction is controlled by the distribution coefficients of the analytes between PDMS and water ($K_{pdms/w}$) that are strongly correlated to the corresponding octanol-water partition coefficients ($K_{o/w}$) [19]. The theoretical extraction recovery of pyrethroids from aqueous samples by SBSE can therefore be estimated using the following equation [19]:

$$\eta = \frac{1}{1 + \beta / K_{o/w}}$$

The theoretical recoveries for a 10 mL water sample and using a stir bar containing 25 µL PDMS (1 cm x 0.5 mm) are all close to 100%. These theoretical recoveries are, however, only obtained after reaching full equilibrium, and possible adsorption effects on the wall of the vial are not taken into account. In the past, low recoveries for highly apolar compounds such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) have been observed when working with SPME and SBSE due to adsorption on glass walls [34, 39-41]. Because the pyrethroids exhibit log $K_{o/w}$ values larger than 5.6, the same behaviour could be expected. A series of experiments was therefore performed using methanol addition to reduce wall adsorption. Different volumes of methanol were added to the sample (10 mL spiked at 0.1 ppb), varying from 0 to 3 mL. After extraction, the stir bars were

analyzed by thermal desorption in the dedicated thermal desorption unit. The results, expressed as relative recoveries normalized to 2 mL methanol addition, are shown in **Figure III.4**. The extractions were at 60 min, 25°C and 900 rpm.

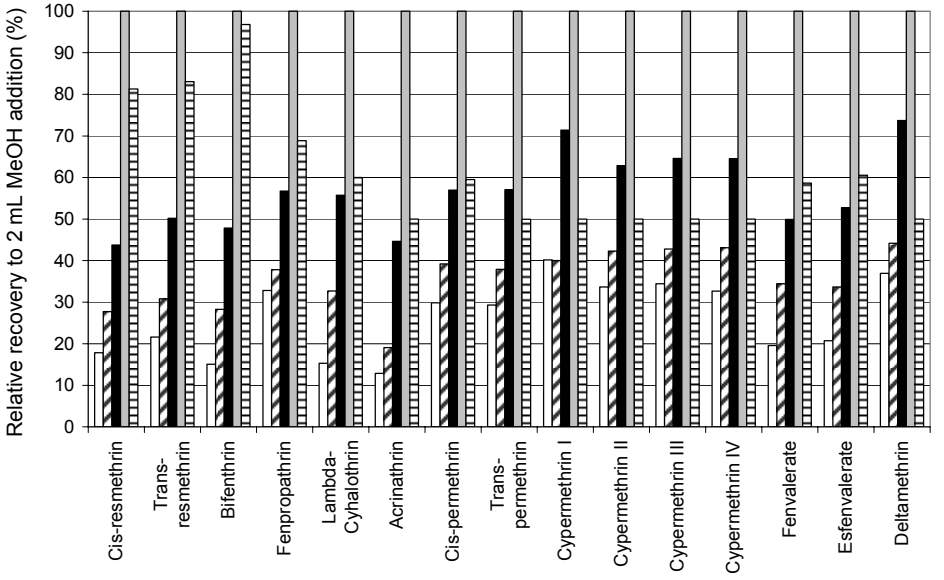


Figure III.4: Effect of MeOH addition on the recovery of the pyrethroids by SBSE-TD(TDS)-GC-MS(SIM): 0 mL (□), 0.5 mL(▨), 1 mL (■), 2 mL (▤) and 3 mL (▤) MeOH.

The addition of methanol increases the recoveries of all pyrethroids significantly and the highest recoveries are obtained at 2 mL methanol concentration.

The influence of the extraction time was evaluated using a constant sample volume of 10 mL with 2 mL methanol addition at the 100 ng/L spiking level. Extraction times of 30, 60, 180 and 360 min were compared. The recovery versus extraction time plots, presented in **Figure III.5**, showed that equilibrium conditions were reached only after 180 min for all pyrethroids with the exception of Bifenthrin for which equilibrium was only reached after 360 min. For practical reasons, however, non-equilibrium conditions were applied and a sampling time of 60 min was selected as an acceptable compromise between total analysis time and sensitivity.

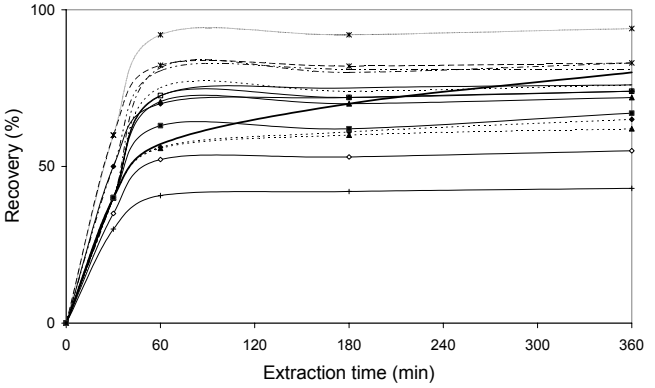


Figure III.5: Influence of the extraction time on the recovery of the pyrethroids by SBSE-TD(TDS)-GC-MS(SIM): cis-resmethrin (—◆—), trans-resmethrin (··▲··), bifenthrin (▬), fenpropathrin (—✳—), λ-cyaholthrin (··◆··), acrinathrin (—+—), cis-permethrin (—), trans-permethrin (----), cypermethrin I (- - -), cypermethrin II (—□—), cypermethrin III (—△—), cypermethrin IV (- ··-), fenvalerate (—✕—), esfenvalerate (—■—) and deltamethrin (—◇—).

2.3.2 Thermal desorption in a dedicated thermal desorption unit

The recoveries obtained by SBSE under the selected conditions i.e. 10 mL sample, 2 mL methanol and stirring at 25°C for 60 min at 900 rpm followed by thermal desorption in a dedicated thermal desorber were measured by comparison of the peak area of the solutes for a 100 ng/L spiked water sample with those obtained by direct liquid injection of 1 µL of a 1 mg/L pyrethroid mixture (1 ng injected). The experimental recoveries are enlisted in **Table III.2**.

Table III.2: Experimental recoveries for the different SBSE methods under optimized conditions.

Pyrethroids	Thermal desorption (%)		Liquid desorption (%)
	TDS	S/SL inlet	
Cis-resmethrin	70.0	33.7	45.5
Trans-resmetrhin	55.8	27.5	28.9
Bifenthrin	57.1	32.0	30.9
Fenpropathrin	92.0	41.9	58.9
λ-Cyhalothrin	56.1	21.3	31.9

Pyrethroids	Thermal desorption (%)		Liquid desorption (%)
	TDS	S/SL inlet	
Acrinathrin	40.7	12.9	29.0
Cis-Permethrin	72.5	26.1	33.2
Trans-permethrin dimethyl d_6 (I.S.)	72.5	20.4	37.8
Trans-permethrin	75.1	20.7	38.2
Cypermethrin I	81.5	22.0	62.4
Cypermethrin II	72.6	19.6	53.6
Cypermethrin III	70.7	18.6	52.1
Cypermethrin IV	80.5	20.6	52.4
Fenvalerate	82.3	21.1	49.9
Esfenvalerate	63.0	14.9	34.4
Deltamethrin	52.2	8.7	33.6

The linearity was evaluated by extracting spiked water samples at 8 concentration levels (1, 2.5, 5, 10, 25, 50, 100, 200 ng/L). The calibration curves were obtained by plotting the peak ratios (pyrethroid/trans-permethrin-d_6) versus the concentrations. All investigated pyrethroids showed good linearity (R^2 values between 0.981 and 0.997) in the investigated range (**Table III.3**). The repeatability was evaluated by analyzing six water samples spiked at 100 ng/L (n=6). The relative standard deviations are included in **Table III.3**. The RSDs are in the range of 4-15%.

Table III.3: Performance of the SBSE methods under optimized conditions: Linearity and repeatability.

Pyrethroids	R^2	Repeatability (%)		
		TDS	S/SL	LD
Cis-resmethrin	0.990	14	12	5
Trans-resmetrhin	0.981	14	18	3
Bifenthrin	0.997	5.6	19.8	9.2
Fenpropathrin	0.994	6.8	11.4	8.4
λ-Cyhalothrin	0.992	12.2	9.9	7.0
Acrinathrin	0.990	3.9	6.5	7.7
Cis-permethrin	0.990	9.4	11.6	8.7

Pyrethroids	R^2	Repeatability (%)		
		TDS	S/SL	LD
Trans-permethrin	0.993	4.2	9.4	9.7
Cypermethrin I	0.997	3.8	9.5	6.1
Cypermethrin II	0.991	4.6	7.1	6.5
Cypermethrin III	0.995	9.4	5.3	4.9
Cypermethrin IV	0.995	5.6	9.3	10.3
Fenvalerate	0.993	10.7	8.6	9.5
Esfenvalerate	0.990	3.9	9.5	10.4
Deltamethrin	0.990	4.7	7.3	9.9

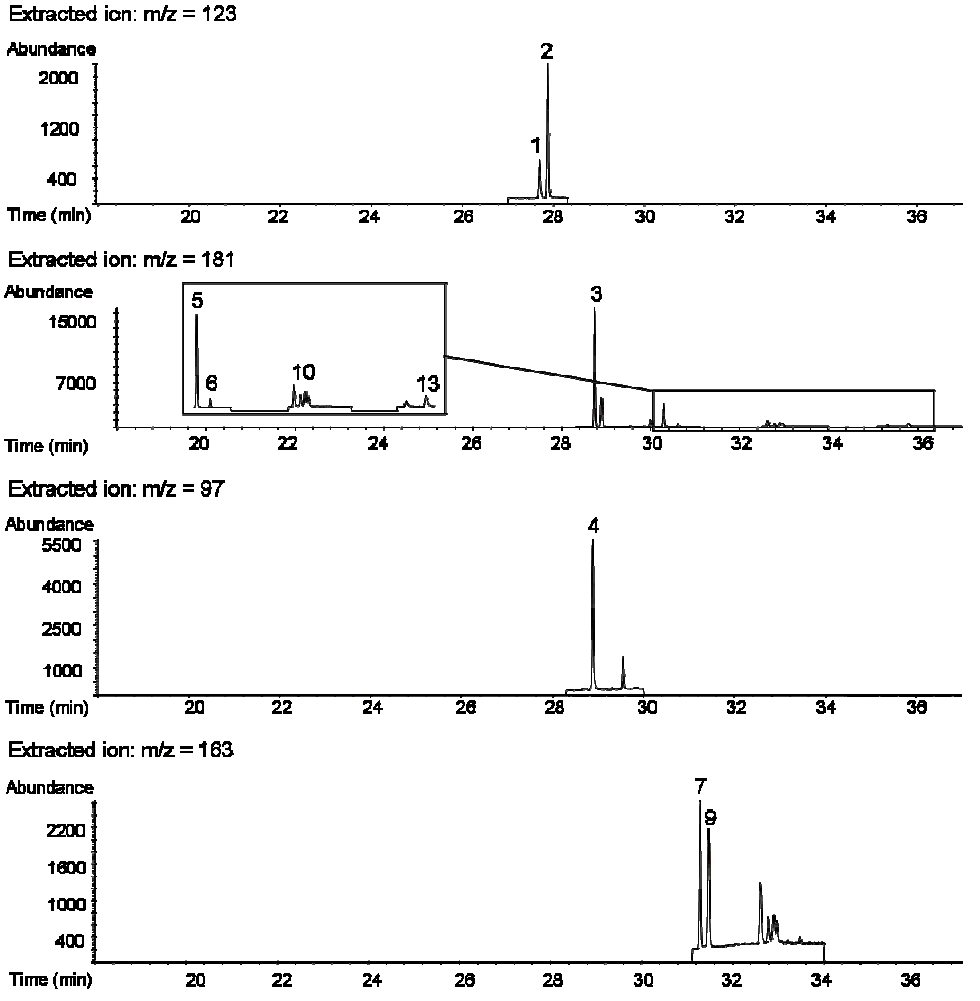
The sensitivity, expressed as limits of detection (LOD) at S/N 3 and limits of quantification (LOQ) at S/N 10, are demonstrated in **Table III.4**. The LOD values are below 1 ng/L (ppt) for all solutes, except for deltamethrin (1.4 ng/L), showing excellent sensitivity of the method.

Table III.4: Performance of the SBSE methods under optimized conditions: Limit of detection and limit of quantitation**.**

Pyrethroids	LOD (ng/L)				LOQ (ng/L)
	TDS	S/SL	LD (1 µL)	LD (10 µL)	TDS
Cis-resmethrin	0.1	0.5	20	2.2	0.4
Trans-resmetrhin	0.2	0.7	60	5.8	0.5
Bifenthrin	0.02	0.09	8	0.9	0.05
Fenpropathrin	0.3	1.3	68	6.6	1.1
λ-Cyhalothrin	0.1	0.6	24	2.5	0.3
Acrinathrin	0.9	3.0	193	19.5	2.9
Cis-Permethrin	0.8	1.2	260	25	2.6
Trans-permethrin	0.6	1.6	175	17.6	1.9
Cypermethrin I	0.2	1.6	36	3.8	0.6
Cypermethrin II	0.2	1.6	43	4.8	0.6
Cypermethrin III	0.2	1.1	52	5.3	0.6
Cypermethrin IV	0.2	1.8	38	3.9	0.7
Fenvalerate	0.3	2.0	73	7.5	1.0
Esfenvalerate	0.8	3.4	221	22.4	2.7

	LOD (ng/L)				LOQ (ng/L)
Deltamethrin	1.4	6.4	320	32.5	4.7

Figure III.6 shows ion chromatograms from the analysis of a 10 mL water sample spiked at the 20 ng/L level. The different pyrethroids can easily be detected. Note that cypermethrin (peak 10) has three chiral centres and thus 8 stereoisomers, or 4 pairs of enantiomers. Cypermethrin will thus give up to 4 peaks in the chromatogram on the apolar column.



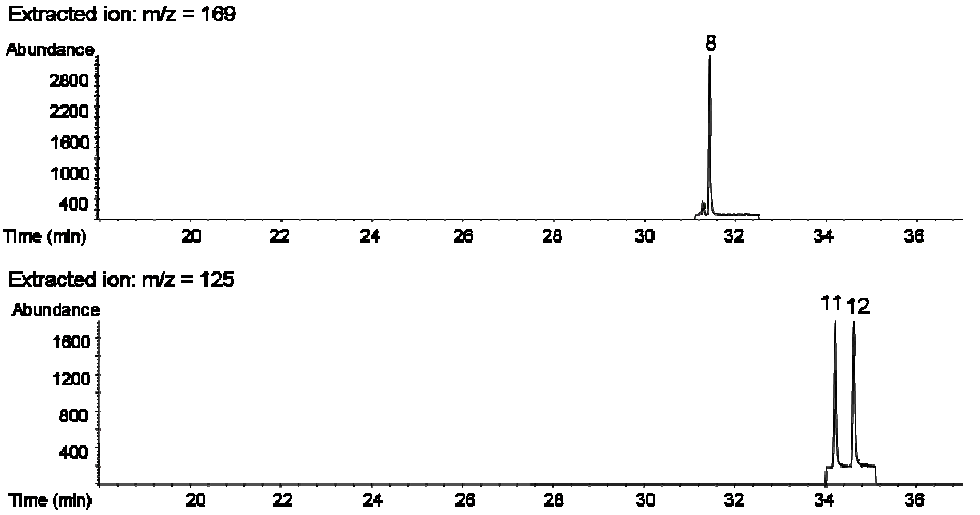


Figure III.6: Extracted ion chromatograms of the pyrethroids from an assay on a 10 mL water sample spiked at 20 ng/L (ppt) level by SBSE-TD(TDS)-GC-MS(SIM). The pyrethroids in this chromatogram are cis-resmethrin (1), trans-resmethrin (2), bifenthrin (3), fenpropathrin (4), λ-cyhalothrin (5), acrinathrin (6), cis-permetrhin (7), trans-permethrin-d6 (8), trans-permethrin (9), cypermethrin (10), fenvalerate (11), esfenvalerate (12), deltamethrin (13).*

2.3.3 Thermal desorption in a split/splitless liner

Thermal desorption in the split/splitless inlet was compared to thermal desorption in the dedicated thermal desorption unit using the same methodology (spiking at 100 ng/L, 60 min extraction time, 900 rpm, 25°C, 10 mL water, 2 mL methanol, n=6). The recoveries are included in **Table III.2** and it is obvious that much lower values are obtained. For deltamethrin, for instance, desorption in a TDS system gave a ca. 6 time higher recovery than desorption in a split/splitless inlet. Moreover, the higher the boiling point, the larger the difference is between the two thermal desorption methods. From this observation, it is clear that desorption in a dedicated thermal desorption unit is more efficient due to adjustable desorption flow and temperature. After introduction of the stir bar in the split/splitless inlet, thermal desorption immediately takes place inside the liner. If desorption is done in splitless mode (for

* A few unlabeled peaks are also present in the different chromatograms. They originate from the aqueous sample that was spiked with the pyrethroids and then analysed using the presented method.

trace analysis), the splitless time is a very important parameter that has a significant influence on the recovery of the pyrethroids. When the purge vent was opened after 1 or 2 min (normal for splitless injection of a liquid sample), the recoveries were very low. Fenvalerate, esfenvalerate and deltamethrin could even not be detected at all. Therefore the splitless time was increased to 5 min. During the time of desorption, the compounds are focussed in the inlet part of the column at initial oven temperature. If no cryogenic cooling is used, the focussing is only effective for compounds with a high boiling point, such as the pyrethroids. Thermal desorption in a standard split/splitless inlet will be difficult to apply to low boiling compounds. However, for high boiling point compounds desorption in a split/splitless liner suffers from another problem. Although desorption took place for 5 min at 300°C before the split valve was activated, this time is too short for complete desorption of these compounds. The main reason for this is the low desorption flow rate (desorption flow rate = column flow = 1.5 mL/min) while a high flow (100 mL/min) was used in the TDS system accompanied by focussing the solutes in the cold CIS-4. The sensitivity decrease (**Table III.2**) compared to TDS is completely reflected by the lower recovery while the repeatability of the split/splitless method at the 100 ng/L level is in the same order as for the TDS experiments (**Table III.3**).

One additional problem encountered with thermal desorption in a split/splitless inlet is column deterioration. When the flip-top system is opened, the liner is filled with air and the oxygen can cause damage to the column and shortens its life time. A higher column bleeding was observed using sequences of thermal desorption using the split/splitless inlet.

2.3.4 Liquid desorption

The procedure for liquid desorption was performed on 10 mL of an aqueous solution spiked at 10 µg/L. Other conditions for extraction were the same as for thermal desorption i.e. 2 mL methanol addition, 25°C, 60 min, 900 rpm. Different solvents (acetone, acetonitrile and ethyl acetate) were tested at 30 min extraction time (1 µL injection) and the results are summarized in **Figure III.7**.

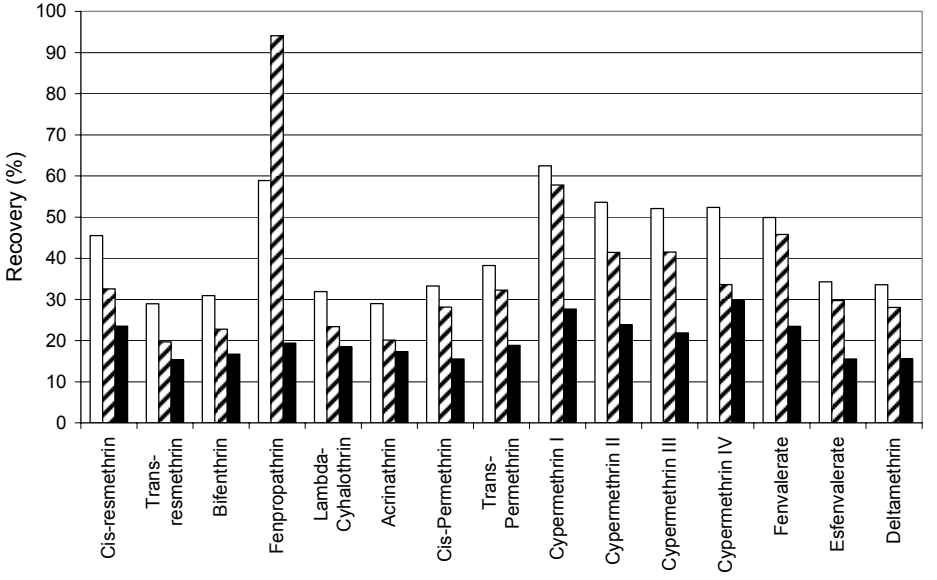


Figure III.7: Influence of different solvents for liquid desorption on the recovery of the pyrethroids by SBSE-LD-GC-MS(SIM): ethyl acetate (□),acetonitrile (▨), acetone (■).

For most pyrethroids, the highest recoveries were obtained using ethyl acetate. The only exception was fenpropathrin for which acetonitrile was the best desorption solvent. Ethyl acetate was selected for further experiments. The time for ethyl acetate extraction was evaluated between 15 and 60 min (**Figure III.8**).

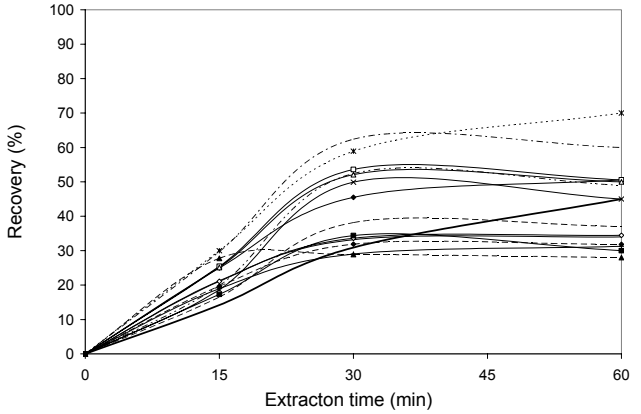


Figure III.8: Influence of the time for ethyl acetate extraction on the recovery of the pyrethroids by SBSE-LD-GC-MS(SIM): cis-resmethrin (—◆—), trans-resmethrin (--▲--), bifenthrin (▬), fenpropathrin (···✳···), λ-cyaholthrin (--◆--), acrinathrin (—+—), cis-permethrin (——), trans-permethrin (----), cypermethrin I (- - - -), cypermethrin II (—□—), cypermethrin III (—△—), cypermethrin IV (- - - -), fenvalerate (—✳—), esfenvalerate (—■—) and deltamethrin (—◇—).

For most of the pyrethroids the amount extracted did not increase using extraction times higher than 30 min. Only for fenpropathrin and bifenthrin were the recoveries slightly higher at 60 min. A desorption time of 30 min was selected for further experiments.

2.3.5 Validation of the method

The recoveries obtained by liquid desorption were calculated by comparing the peak area after liquid desorption and 1 µL injection with those obtained for 1 µL direct injection of a 10 µg/L solution in ethyl acetate. The recoveries are listed in **Table III.2**. The values in the order of 29-62% are between the values obtained by thermal desorption in the SSL inlet and in the dedicated thermal desorber.

The repeatability of liquid desorption, measured at the 1 ppb level (n=6), is listed in **Table III.3** and was similar to the values obtained by the thermal desorption methods. The sensitivity of the liquid desorption method was calculated for 1 and 10 µL injections (**Table III.4**). Compared to thermal desorption, the LOD values are much higher due to the dilution effect. Only 0.67 % and 6.7 % of the extracted amount is injected for 1 and 10 µL injections, respectively. It is clear that liquid desorption

should preferentially be used in combination with large volume injection. Larger injection volumes were not evaluated in this work because this also requires dedicated instrumentation.

2.4 Conclusions

Stir bar sorptive extraction (SBSE) with thermal or liquid desorption in combination with retention-time-locked GC is a versatile method for the determination of the pyrethroids in water samples. Methanol was added to the sample solutions to minimize the effect of glass adsorption. The developed methods were reproducible and sensitive. All three desorption methods have shown to be able to detect the pyrethroids in compliance with European regulations. Thermal desorption in a split/splitless inlet equipped with a flip-top device is a valuable alternative when a dedicated thermal desorption unit is not available. The method is, however, less sensitive than the TDS method, but is still much more sensitive than liquid desorption. The lower sensitivity in slit/splitless desorption is accompanied by a boiling point discrimination effect and both are due to a lower desorption flow and the lack of cryofocussing. Finally, also liquid desorption can be applied, but to reach good sensitivity at least 10 µL injections are required.

3 Development of a multi-residue method for the determination of EDCs in aqueous samples*

3.1 Introduction

Stir bar sorptive extraction in combination with thermal desorption and GC-MS analysis is the most suitable technique to develop a multi-residue method for the determination of EDCs in aqueous samples.

Since the apolar PDMS phase is not highly effective to extract highly polar compounds, *in-situ* derivatization was carried out. *In-situ* derivatization not only improves the effectiveness of the PDMS phase in analyte recovery, but also the gas chromatographic analysis.

The heterogeneous group of EDCs can be divided into five different sub-groups, based on their functionality. The first group contains phenolic EDCs. They are very easily acylated when using acetic acid anhydride as *in-situ* derivatization reagent [25,30,31,42] (**Figure III.9**).

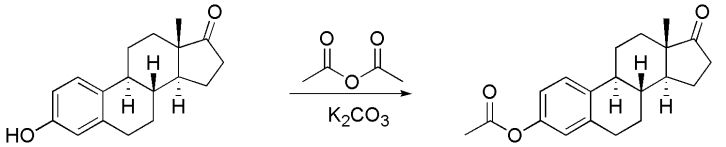


Figure III.9: Derivatization of phenolic EDCs using acetic acid anhydride.

The amine-based EDCs belong to the second group. As reported by Katoaka [43], ethyl chloroformate can be used for the conversion of primary amines to their corresponding ethyl carbamates (**Figure III.10**). The third group includes the acidic

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* *Published as:*
'Multi-residue screening of endocrine disrupting chemicals and pharmaceuticals in aqueous samples by multi-stir bars sorptive extraction – single desorption – capillary gas chromatography/mass spectrometry',
E. Van Hoeck, F. Canale, C. Cordero, S. Compernolle, C. Bicchi, P. Sandra, Anal. Bioanal. chem., DOI 10.2007/s00216-008-2339-7.
'Towards automated, miniaturized and solvent-free sample preparation methods'
F. David, E. Van Hoeck, P. Sandra, Anal. Bioanal. Chem. 387 (2007) 141

EDCs. According to Husek, the use of ethyl chloroformate in the presence of ethanol transforms them into their less polar ethyl derivatives [44] (**Figure III.10**). The latter two groups can thus be derivatized with the same procedure.

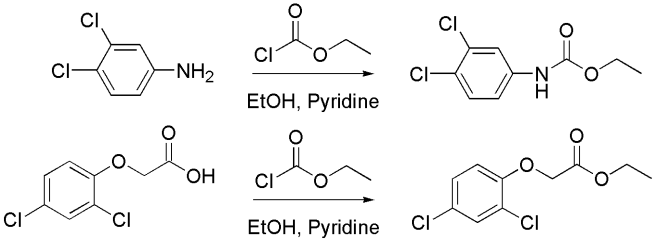


Figure III.10: Derivatization of acidic EDCs using ethyl chloroformate.

The fourth group consists of the organotin compounds. The most effective derivatisation for these chemicals involves the use of sodium tetraethylborate ($NaBEt_4$), hereby converting the organotin species into ethyl derivatives [45] (**Figure III.11**).

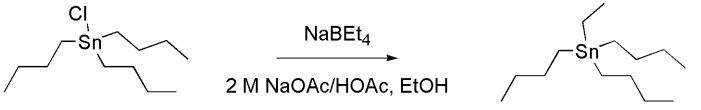


Figure III.11: Derivatization of organotin compounds using sodium tetraethylborate.

The last group incorporates EDCs with log $K_{o/w}$ values > 5 and addition of an organic modifier like methanol to the sample is required to reduce wall adsorption effects [34,38,39,46]. After sampling, the four stir bars are all introduced together with a plug of glass wool impregnated with BSTFA in one thermal desorption tube and analysed simultaneously by capillary GC-MS. Several parameters that influence the *in-situ* derivatization or extraction efficiency were tested. The method was developed on some representative EDCs for each group using labelled internal standards and then extended to a larger number of them and of pharmaceuticals as well. The performance was evaluated in terms of linearity, repeatability and limits of detection (LODs). At last, the method was applied to screen and quantify EDCs and some pharmaceutical residues in process waters and hospital effluent water.

3.2 Experimental

3.2.1 Chemicals

Neat certified endocrine disrupting standards were purchased from different sources. 4-n-nonylphenol (purity 98.4%), atrazine (99%), alachlor (99.9%), 3.4-dichloroaniline (99.3%) and 7.12-dimethylbenzo(a)anthracene (99.4%) were purchased from Riedel de Haën (Seelze, Germany). Tripropyltinchloride (99.5%) was purchased from Dr. Ehrenstorfer (Augsburg, Germany). 2.4-dichlorophenol (99%), 4-t-butylphenol (99%), propofol (97%), 4-n-octylphenol (99%), bisphenol A (99%), tributyltinchloride (96%), 2.4-D (98%), 17-β-estradiol-d_3 (98%) and anthracene-d_{10} (98%) were supplied by Aldrich (Bornem, Belgium). Estrone (99%), 17-β-estradiol (98%), 2.4.5-T (97%) and 4-n-nonylphenol-d_4 (97%) were purchased from Sigma (Bornem, Belgium). Triphenyltinchloride (97%), bis(2-ethylhexyl)phthalate (97%) and benzo(a)pyrene were supplied by Fluka (Bornem, Belgium). 3.4-dichloroaniline-ring-$^{13}C_6$ (99%) was supplied by Cambridge Isotope Laboratories (LGC Promochem, Teddington, UK). Triphenyltinchloride-d_{15} was received from the Research Institute for Chromatography (Kortrijk, Belgium). Cholesterol (99%), coprostanol (98%), β-sitosterol (90%), stigmasterol (90%), dibutylphthalate (98%), diethylphthalate (96%), ketoprofen, naproxen (98%) and ibuprofen (98%), were from Sigma (Milan, Italy). The chemical structures of the endocrine disrupting chemicals are given in **Figure III.12**. The EDCs adopted for model standard solutions are underlined.

Stock solutions of each individual compound were prepared in acetone at a concentration of 1000 mg/L. The solutions were stored at 4°C and used to prepare the spiking solutions.

Acetic acid anhydride (AAA), ethyl chloroformate (ECF), N.O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), potassium carbonate (K_2CO_3), potassium phosphate monobasic (KH_2PO_4), potassium phosphate dibasic (K_2HPO_4), methanol (MeOH), ethanol (EtOH), acetic acid (HOAc), sodium acetate (NaOAc), acetone and sodium tetraethylborate ($NaEt_4B$) were from Sigma (Bornem, Belgium).

Water samples used for method development were obtained by a Milli-Q RG system (Millipore, Molsheim, France) in agreement with the ISO 9002 Quality Systems Standards.

Real-world water samples consist in a set of surface waters collected from a water treatment plant and were kindly supplied by Prof. Giorgio Gilli, Dipartimento di Sanità Pubblica e Microbiologia, University of Turin (Turin, Italy). Hospital effluent water was obtained from the Ghent University hospital, Belgium.

3.2.2 Sample preparation

Commercial stir bars for sorptive extraction (Twister^{TM}) were purchased from Gerstel (Gerstel GmbH, Mülheim an der Ruhr, Germany). They consist of a 10 mm length glass-encapsulated magnetic stir bar, coated with 25 µL of PDMS (0.5 mm coating). Stir bars were conditioned for 2 h at 300°C under a constant helium flow and kept in 2 mL vials before use as indicated by the manufacturer. The stir bars have been used more than 50 times after appropriate re-conditioning.

The derivatization reactions and the addition of methanol were optimized on water samples of 10 mL, spiked with 10 µL of a 1/10/50 µg/mL EDC standard solution for the analysis in scan mode and 0.1/1/5 µg/mL solution for the analysis in SIM mode. The water samples were also spiked with 10 µL of the deuterated internal standard solution in the same concentration range. The spiked water sample contained 2.4-dichlorophenol, 4-t-butylphenol, 4-n-octylphenol, bisphenol A and bis(2-ethylhexyl)phthalate in a concentration of 1 ng/mL for scan mode and 0.1 ng/mL for SIM mode. 4-n-nonylphenol and 4-n-nonylphenol-d_4 were present at a 10 ng/mL concentration for scan mode and at 1 ng/mL for SIM mode. Estrone, alachlor, tripropyltinchloride, tributyltinchloride, triphenyltinchloride, benzo(a)pyrene, anthracene-d_{10} and 7.12-dimethylbenzo(a)anthracene were at 10 ng/mL in scan mode and at 1 ng/mL in SIM mode. 3.4-dichloroaniline and 3.4-dichloroaniline-ring-$^{13}C_6$ concentrations were 20 ng/mL for the analysis in scan mode and 2 ng/mL in SIM mode. At last, atrazine, 17-β-estradiol, 17-β-estradiol-d_3, 2.4-D and 2.4.5-T were at 50 ng/mL for scan mode and at 5 ng/mL for SIM mode.

The first derivatization reaction was performed by adding 0.5 g K_2CO_3 and 500 µL acetic acid anhydride, hereby converting the phenolic compounds to their corresponding acetates. The reaction is excellent for all phenolic compounds, with the exception for 17-β-estradiol. To improve its analysis, this compound was further derivatized with an in-tube silylation with 1 µL BSTFA. For the second aliquot, 100 µL ethyl chloroformate and 300 µL ethanol were added to the aqueous solution to derivatize amine-based and acid-based EDCs simultaneously. Different buffers leading to different pH values were tested to maximise the conversion of acids and primary amines into the corresponding ethyl esters and ethyl carbamates, respectively. The buffers were 0.5 g potassium phosphate monobasic (pH 5), 200 µL pyridine (pH 8), 0.5 g potassium phosphate dibasic (pH 9), and 0.5 g potassium carbonate (pH 11). The derivatization efficiency of these buffers was compared to that obtained without addition. For the third aliquot, 300 µL 1% sodium tetraethylborate solution in water was used to ethylate organotin compounds. To avoid adsorption of these compounds onto the glass-wall or other particles, 1 mL ethanol was always added to the aqueous solution. Similarly to the derivatization with ethyl chloroformate, different buffers were tested i.e. sodium acetate/acetic acid (pH 5), 0.2 g potassium phosphate monobasic (pH 5), 0.5 g potassium phosphate monobasic (pH 5) and 0.5 g potassium carbonate (pH 11). This procedure has also been carried out without adding a buffer. At last, the extraction of the very apolar compounds was improved by adding methanol in amounts ranging from 0 to 3 mL to reduce the adsorption onto the vial glass-wall. The four optimised extraction procedures were then combined, to develop a multi-residue method for the determination of EDCs in aqueous solutions. Four aliquots of 10 mL of a given aqueous sample were taken. Each aliquot was submitted to a different optimised extraction at RT while stirring at 900 rpm (Variomag Multipoint 6/15, H+P Labortechnik, Oberschleissheim, München, Germany) for an extraction time of 60 min. Afterwards, the stir bars were removed from the aqueous solution with tweezers and dried on a lint-free tissue. The four stir bars were then all placed in one thermal desorption tube together with 1 µL of BSTFA and analysed simultaneously by capillary GC-MS.

The optimised extraction procedures were then applied to other EDCs and pharmaceuticals. The method was finally used to screen real-world water samples. These samples (4x10 mL) were spiked with 10 µL of each internal standard spiking solution.

3.2.3 Instrumental

An Agilent 6890 gas chromatograph – 5973 mass spectroscopic detector combination (Agilent Technologies, Little Falls, DE, USA) equipped with a PTV inlet (CIS4, Gerstel GmbH, Mullheim, Germany) was used. Thermal desorption was carried out in a TDS unit (TDS-2, Gerstel GmbH, Mullheim, Germany) assembled on the GC unit via the PTV inlet. The stir bars were placed into a glass tube of 187 mm L, 6 mm OD and 4 mm ID in the thermal desorption unit. Splitless thermal desorption was performed by programming the TDS from 35°C (1 min) to 300°C (5 min) at a rate of 60°C/min with a helium flow rate of 100 mL/min. The analytes were cryo-focussed in the PTV inlet at -150°C using liquid nitrogen. Splitless injection was performed by ramping the PTV from -150°C (0.10 min) to 300°C (5 min) at a rate of 10°C/s. Capillary GC analyses were carried out on an HP-5MS fused silica capillary column (5% diphenyl, 95% dimethylsiloxane) of 30 m L, 0.25 mm ID and a film thickness of 0.25 µm (Agilent Technologies, Folsom, USA). The oven was programmed from 70°C (2 min) to 150°C at 25°C/min, then to 200°C at 3°C/min and finally at 8°C/min to 280°C (10 min). Helium in constant pressure mode was used as carrier gas. The head pressure was adjusted using the retention time locking (RTL) software (Agilent Technologies) to obtain a constant retention time of 19.23 min for chlorpyrifos.

Detection was carried out with the MS in scan or SIM mode. The transfer line, ion source and quadrupole analyser temperatures were maintained at 280°C, 230°C and 150°C, respectively, and a solvent delay of 4 min was used. Electron ionisation mass spectra were recorded at 70eV electron energy with an ionisation current of 34.6 µA. The target ions, used for quantification are listed in **Table III.5**. Data acquisition, instrument control and data analysis were performed by the MSD ChemStation software (G1701CA-version C.00.00, Agilent Technologies).

3.3 Results and discussion

3.3.1 Optimization of the extraction procedure

Multi-residue SBSE method development focussed in first instance on chemical classes of analytes belonging to the EDCs. As a consequence a set of compound(s) representative of each class was chosen and the most suitable tailored sample preparation method was developed to enhance their PDMS affinity and recovery and to improve their chromatographic properties. The structures of all the EDCs used in this study are given in **Figure III.12**. The model analytes, each representing one of the five classes of EDCs, could be divided into four groups coherent with the principles cited above: the first one included phenolic compounds (2,4-dichlorophenol, 4-t-butylphenol, 4-n-octylphenol, 4-n-nonylphenol, bisphenol A, estrone, 17-β-estradiol), the second one includes both amines and acids (2,4-D, 2,4,5-T, 3,4-dichloroaniline), the third one the organotin derivatives (tripropyltinchloride tributyltinchloride, triphenyltinchloride) and the fourth one apolar and/or not derivatizable polar compounds (bis(2-ethylhexyl)phthalate, 7,12-dimethylbenzo- (a)anthracene, benzo(a)pyrene, alachlor, atrazine). **Table III.5** shows the log $K_{o/w}$ values, the theoretical recovery, the derivatization reagent effective for the specific EDC, the locked retention time and the target ion for quantitation together with the qualifier ions. The table is extended with solutes identified in waters samples during the study.

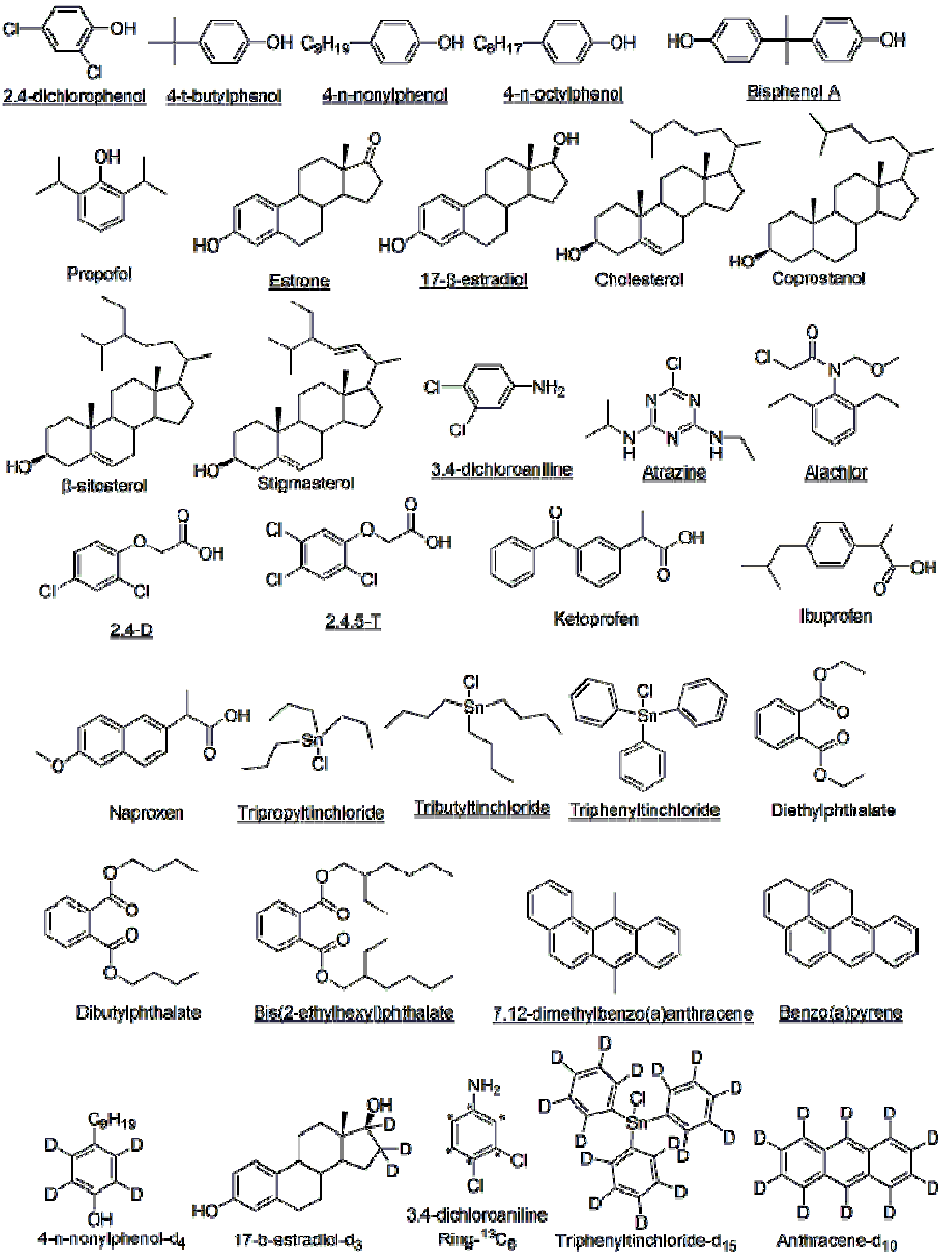


Figure III.12: Structures of the endocrine disrupting chemicals and pharmaceuticals investigated. The EDCs used for the development of the method are underlined.

Table III.5: Summary of EDCs and pharmaceuticals used in method development and detected in this study; Log $K_{o/w}$ value; derivatization agent; locked retention times (min) of derivatives; target and qualifiers ions selected for SIM mode acquisition.

Reference EDCs and pharmaceuticals	Log $K_{o/w}$*	Theoretical recovery (%)	Derivatization and extraction		
			Derivatizing Reagent	Retention time (min)	Target ion$ and Qualifiers
2,4-dichlorophenol	2.80	60.2	AAA	6.76	63, *162*, 164
4-t-butylphenol	3.42	86.3	AAA	7.18	107, *135*, 150
4-n-octylphenol	5.50	99.9	AAA	15.83	*107*, 108, 206
4-n-nonylphenol-d_4	/	/	AAA	18.53	*111*, 112, 224
4-n-nonylphenol	5.99	100	AAA	18.56	*107*, 108, 220
Bisphenol A	3.64	91.3	AAA	27.27	*213*, 228, 270
Propofol	3.57	89.9	AAA	7.25	*163*, 178, 220
Estrone	3.43	86.6	AAA	31.86	185, *270*, 271
17-β-estradiol	3.94	95.4	AAA/BSTFA	32.39	213, 254, *344*
17-β-estradiol-d_3	/	/	AAA/BSTFA	32.37	73, 213, *347*
Ibuprofen	3.79	93.7	ECF	10.15	191, *161*, 117
2,4-D	2.62	50.0	ECF	12.04	*175*, 185, 248
3,4-dichloroaniline	2.37	36.0	ECF	15.02	*161*, 187, 233
3,4-dichloroaniline-$^{13}C_6$	/	/	ECF	15.02	*167*, 193, 239
2,4,5-T	3.26	81.4	ECF	15.43	*209*, 211, 284
Naproxen	3.10	75.1	ECF	20.91	258, *185*, 141
Ketoprofen	3.00	70.6	ECF	23.91	209, *105*, 77
Tripropyltinchloride	3.23	80.3	$NaEt_4B$	6.04	191, *193*, 235
Tributyltinchloride	4.70	99.2	$NaEt_4B$	8.71	177, 205, *207*
Triphenyltinchloride-d_{15}	/	/	$NaEt_4B$	26.59	362, 364, *366*
Triphenyltinchloride	3.93	95.3	$NaEt_4B$	26.72	347, 349, *351*
Anthracene-d_{10}	/	/	MeOH	14.05	94, *188*, 189
Diethylphthalate	2.65	51.7	MeOH	9.66	222, 176, *149*
Dibutylphthalate	4.61	99	MeOH	18.10	223, 205, *149*
DEHP	8.39	100	MeOH	29.63	57, 167, *149*
7,12-dimethylbenzo[a] anthracene	6.11	100	MeOH	32.32	239, 241, *256*
Benzo[a]pyrene	6.62	100	MeOH	33.41	250, *252*, 253

Reference EDCs and pharmaceuticals	Log $K_{o/w}$*	Theoretical recovery (%)	Derivatization and extraction		
			Derivatizing Reagent	Retention time (min)	Target ion$ and Qualifiers
Alachlor	3.37	84.9	MeOH	17.10	*45*, 160, 188
Atrazine	2.82	61.3	MeOH	13.35	173, *200*, 215
Coprostanol	8.82	100	BSTFA	34.84	388, *373*, 233
Cholesterol	8.74	100	BSTFA	35.44	*386*, 275, 213
Stigmasterol	9.43	100	BSTFA	36.49	*412*, 133
β-sitosterol	9.65	100	BSTFA	37.09	*414*, 105, 145

* Octanol/water coefficients are obtained by the software program SRC-KOWWIN (Syracuse Research, Syracuse, NY, USA)

$ Target ions in *ITALIC*

Standard mix reference compounds are underlined

Abbreviations of the derivatizing agents: Acetic acid anhydride (AAA), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and ethyl chloroformate (ECF)

Each sampling procedure was optimized to enable us to apply only one thermo-desorption step in order to carry out only one chromatographic run to detect and quantify all target compounds investigated. The sample volume was fixed at 10 mL, extraction was performed at 25°C and at 900 rpm. The influence of extraction time was also evaluated by comparing the recovery *vs* extraction time after 30, 60, 180 and 360 min of SBSE extraction. The results showed that for some solutes sampling times longer than 360 min were necessary to obtain equilibrium but, non-equilibrium conditions were chosen for practical reasons. A sampling time of 60 min was adopted as an acceptable compromise between total analysis time and sensitivity.

Each derivatization reaction was then studied and optimised by varying critical parameters.

3.3.1.1 Derivatization with acetic acid anhydride and BSTFA

The derivatization of phenolic compounds with acetic acid anhydride has already extensively been described in the literature [42,47]. This acetylation is mostly carried out with 500 µL acetic acid anhydride and 0.5 g K_2CO_3 for pH correction. The

reaction is excellent for all phenolic EDCs apart from 17-β-estradiol. This chemical has both an aromatic and an aliphatic hydroxyl and acetylation only converts the aromatic hydroxyl group in an acetate group. For this reason, Kawaguchi et al. developed a "dual derivatization" method for determination of 17-β-estradiol by SBSE with *in-situ* acetylation followed by thermal desorption with in-tube silylation [48]. The aromatic hydroxyl was derivatized by *in-situ* acetylation to increase the recovery of 17-β-estradiol while the second derivatization step increased the volatility of the acyl derivative of 17-β-estradiol. The final procedure applied for hydroxylated compounds therefore consists of an *in-situ* derivatization with 0.5 g K_2CO_3 and 500 µL acetic acid anhydride, followed by in-tube silylation with 1 µL BSTFA. All hydroxyl-groups are derivatized by this procedure.

3.3.1.2 Derivatization with ethyl chloroformate

The procedure described by Tienpont et al. for the analysis of biological fluids by SBSE and *in-situ* derivatization was adopted and optimized [49]. In this procedure 200 µL pyridine was used to correct the pH to 8. The influence of pH on the derivatization effectiveness was evaluated by a set of different buffers: 0.5 g potassium phosphate monobasic (pH 5), 0.5 g potassium phosphate dibasic (pH 9), and 0.5 g potassium carbonate (pH 11). The results obtained with these buffers were compared to those obtained without. **Figure III.13** shows the results, expressed as relative peak areas normalized to 0.5 g KH_2PO_4 showing that this is the most effective buffer.

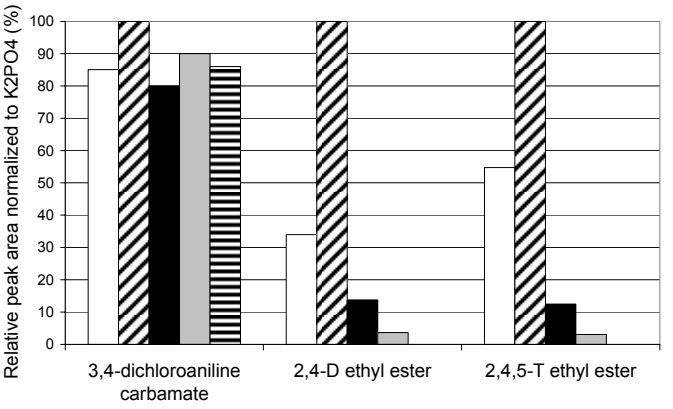


Figure III.13: Influence of the buffer on the derivatization efficiency of the reaction with ethyl chloroformate: Without buffer (□), 0.5 g KH_2PO_4 (▨), 200 µL (■), 0.5g K_2HPO_4 (▩) and 0.5 g K_2CO_3 (▤).

As can be seen from these results, the nature of the buffer had little or no significant influence on the derivatization efficiency of primary amines. The optimized conditions to derivatize primary amines and acids were with 0.5 g KH_2PO_4, 300 µL ethanol and 100 µL ethyl chloroformate.

3.3.1.3 Derivatization with sodium tetraethylborate

The determination of organotin compounds was based on the *in-situ* derivatization combined with SPME-GC-MS method developed by Devos et al. [45]. In analogy with derivatization with ethyl chloroformate, different buffers were tested: 0.2 M sodium acetate/acetic acid (pH 5), 0.2 g potassium phosphate monobasic (pH 5), 0.5 g potassium phosphate monobasic (pH 5) and 0.5 g potassium carbonate (pH 11).

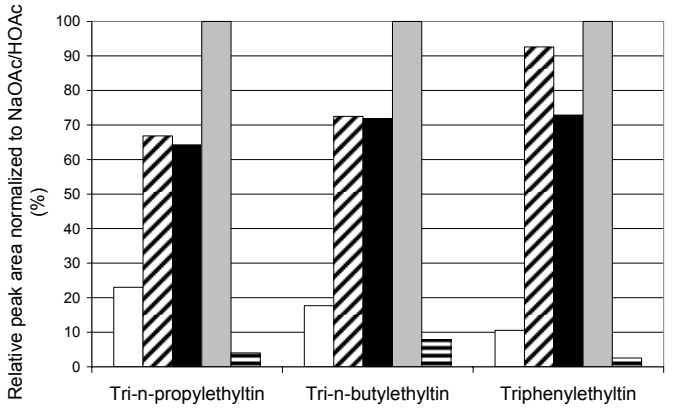


Figure III.14: Influence of the buffer on the derivatization efficiency of the reaction with sodium tetraethylborate: Without buffer (□), 0.2 g KH_2PO_4 (▨), 0.5 g KH_2PO_4 (■), 0.2M NaOAc/HOAc (▩) and 0.5 g K_2CO_3 (▤).

The results, expressed as relative peak areas normalized to sodium acetate/acetic acid addition, are reported in **Figure III.14**, from which it is clear that the highest derivatization efficiency is obtained with the sodium acetate/acetic acid buffer. The sodium acetate/acetic acid buffer was therefore applied together with 1 mL ethanol and 300 µL 1% sodium tetraethylborate in water.

3.3.1.4 Addition of an organic modifier

In the past, limited recoveries for apolar compounds such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) were observed with SPME and SBSE due to their adsorption on glass-walls [34,38,39,46]. Therefore, different volumes of methanol (from 0 to 3 mL) were added to the sample. The results expressed as relative peak area normalized to 2 mL methanol addition are shown in **Figure III.15**.

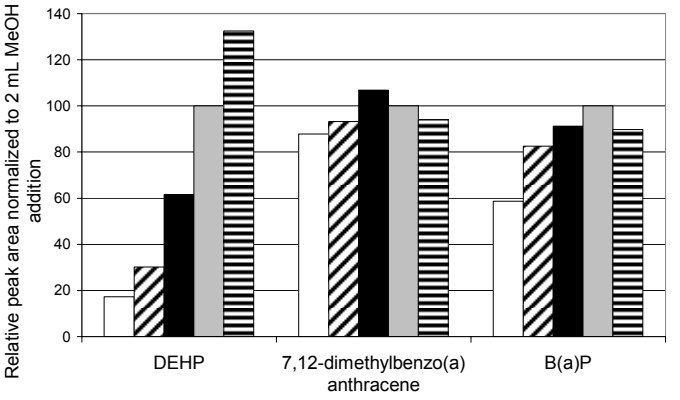


Figure III.15: Influence of the addition of different volumes of methanol on the extraction efficiency of the apolar EDCs: 0 mL MeOH (□), 0.5 mL MeOH (▨), 1 mL MeOH (■), 2 mL MeOH (▩) 3 mL MeOH (▤).

Methanol addition significantly increases the recoveries for all apolar EDCs. In particular, the overall best results were obtained with 2 mL methanol (i.e. 20% MeOH) that was therefore used for the following experiments.

3.3.1.5 Combination of the three derivatization reactions and the methanol addition

Kawaguchi et al. developed, in order to increase the sensitivity, the 'multi-shot' thermal desorption approach, in which up to five stir bars were placed in one thermal desorption tube from where they were simultaneously desorbed and analysed [42]. In the approach described, multi-shot SBSE is used to increase on elucidation and quantitation of as many EDCs and pharmaceuticals as possible. Four aliquots of 10 mL from a given aqueous sample are extracted with one of the optimized extraction procedures. After sampling, the four stir bars and a glass wool plug with BSTFA are placed in a thermal desorption tube and analysed simultaneously by capillary GC-MS analysis. **Figure III.16** shows the set-up of the thermal desorption tube.

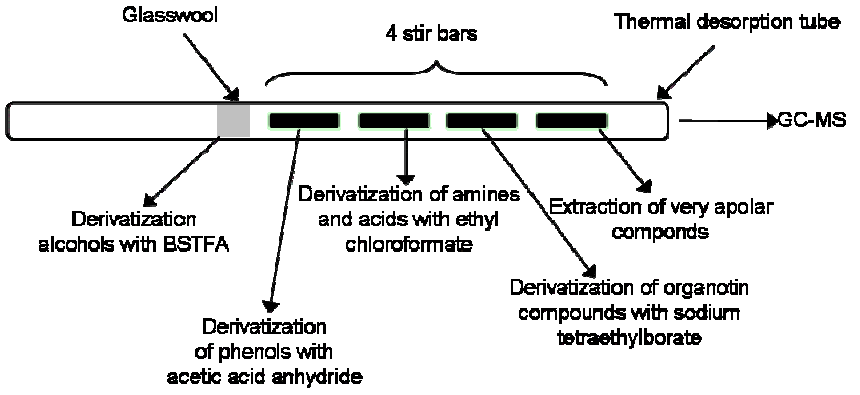
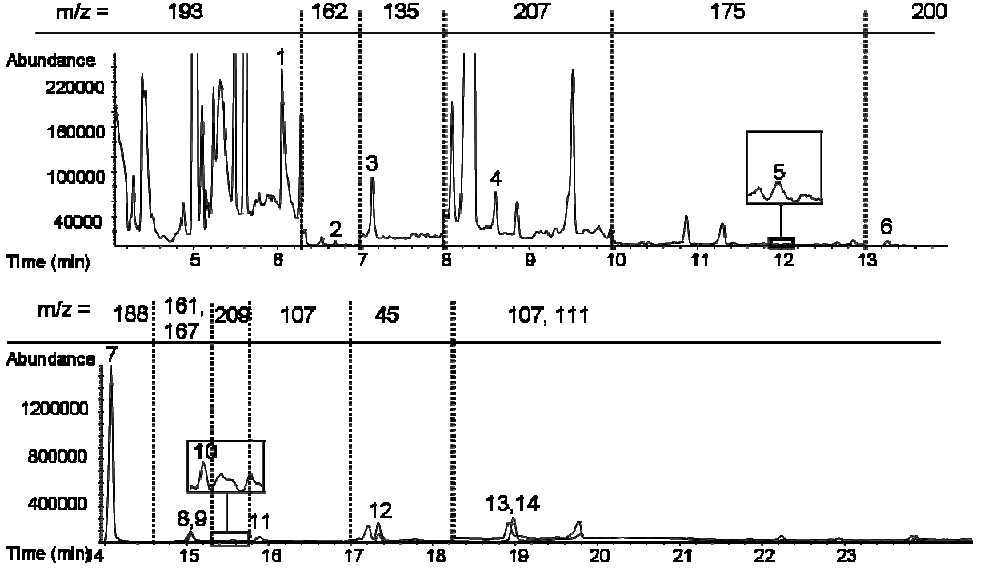


Figure III.16: Set-up of the thermal desorption tube in the multi-shot mode.

3.3.2 Figures of merit of the SBSE method

Figure III.17 shows a combination of the different extracted ion chromatograms from the analysis of a blank water sample with each EDC, used for the optimisation of the method, spiked at a concentration between 0.01 and 0.5 µg/L. The water sample was analysed in agreement with the multi-residue method described above in SIM mode.



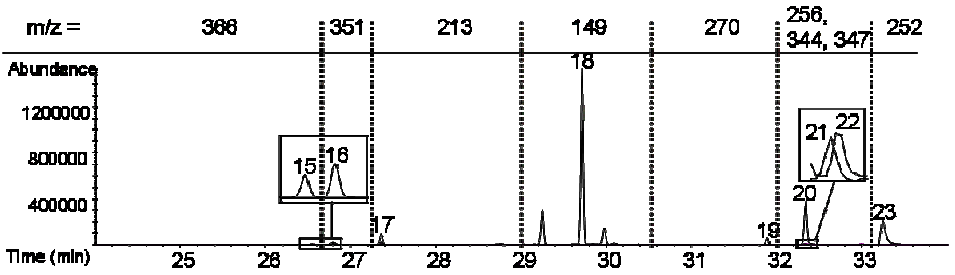


Figure III.17: Combination of the different extracted ion chromatograms in SIM mode. Peak identification: tripropylethyltin (1), 2.4-dichlorophenyl acetate (2), 4-t-butylphenyl acetate (3), Tributylethyltin (4), 2,4-D ethyl ester (5), atrazine (6), anthracene-d_{10} (7), 3,4-dichloroaniline carbamate (8), 3,4-dichloroaniline carbamate-Ring-$^{13}C_6$ (9), 2,4,5-T ethyl ester (10), 4-octylphenyl acetate (11), alachlor (12), 4-n-nonylphenyl acetate (13), 4-n-nonylphenylacetate-d_4 (14), Triphenylethyltin-d15 (15), triphenylethyltin (16), Bisphenol A diacetate (17), DEHP (18), Estrone acetate (19), 7,12-dimethylbenzo(a)anthracene (20), 17-β-(trimethylsiloxy)estradiol acetate-d_3 (21), 17-β-(trimethylsiloxy)estradiol acetate (22), benzo(a)pyrene (23).

Among all the investigated EDCs, only DEHP, 4-t-butylphenol and bisphenol A were found in the blanks in a concentration of 317 ng/L, 2 ng/L and 0.8 ng/L respectively. Linearity was evaluated by extracting spiked water samples at four concentration levels in scan mode and at five concentration levels in SIM mode. The calibration curves were obtained by plotting peak ratios (EDC/EDC deuterated reference) *vs.* concentrations. The results are shown in **Table III.6**. All investigated EDCs showed a good linearity in the investigated ranges (R^2 values between 0.986 and 0.999 in scan mode and between 0.950 and 0.998 in SIM mode).

Table III.6: Linearity, repeatability and detection limits in scan and SIM mode

Reference derivatised EDCs	Linearity (R^2)	Repeatability (%)		LOD (ng/L)	
	SIM	SCAN	SIM	SCAN	SIM
2,4-dichlorophenyl acetate	0.996	9	8	8	0.17
4-t-butylphenyl acetate	0.990	10	8	4	0.10
4-n-octylphenyl acetate	0.991	5	7	15	0.27
4-n-nonylphenyl acetate	0.994	4	9	27	0.57
Bisphenol A diacetate	0.990	10	6	5	0.04
Estrone acetate	0.997	10	10	28	0.33
17-β-(trimethylsiloxy)estradiol acetate	0.995	6	4	422	2.81

Reference derivatised EDCs	Linearity (R^2)	Repeatability (%)		LOD (ng/L)	
	SIM	SCAN	SIM	SCAN	SIM
3,4-dichloroaniline ethyl carbamate	0.999	5	6	55	3.59
2,4-D ethyl ester	0.994	8	11	510	22
2,4,5-T ethyl ester	0.996	12	14	324	3.00
Tripropylethyltin	0.990	12	12	138	0.39
Tributylethyltin	0.996	6	15	190	3.60
Triphenylethyltin	0.995	5	4	24	0.21
Atrazine	0.993	9	12	266	4.55
Alachlor	0.987	14	14	22	1.10
Di(ethylhexyl)phthalate	0.951	10	15	1	0.01
7,12-dimethylbenzo(a)anthracene	0.993	11	8	6	0.82
Benzo(a)pyrene	0.998	14	14	16	0.51

The repeatability was measured by analyzing six spiked water samples. The relative standard deviations for both scan and SIM mode are included in **Table III.6**. For both methods RSDs in the range of 4-14% were found.

Finally, the limits of detection for the target compounds achievable with this method were measured as the concentrations detectable with a signal-to-noise ratio of 3. The LOD values are listed in **Table III.6** and range from 1 ng/L for DEHP to 500 ng/L for 2,4-D in scan mode and from 0.01 ng/L for DEHP to 22 ng/L for 2,4-D in SIM mode.

3.3.3 Analysis of real-world water samples

The effectiveness and robustness of the multi-shot SBSE method have been evaluated by analysing real-world water samples submitted to the total procedure. Twelve samples of effluents resulting from depuration processes in the region of Turin (Italy) and one water sample from the Ghent university hospital (Belgium) were analysed. Note that the hospital has its own waste water treatment plant and the water was collected at the outlet line. It is not the aim of this contribution to discuss all data obtained but rather to illustrate the performance of the multi-residue method.

The derivatization with acetic acid anhydride (AAA) and BSTFA enabled us to identify and quantify 17-β-estradiol (**Figure III.18**a) and bisphenol A (**Figure III.18**b) in

the samples from Torino depuration plants in concentrations of 74 ng/L and 104 ng/L, respectively. Analysis of the hospital effluent water revealed the presence of propofol in a concentration of 310 ng/L (**Figure III.18**c).

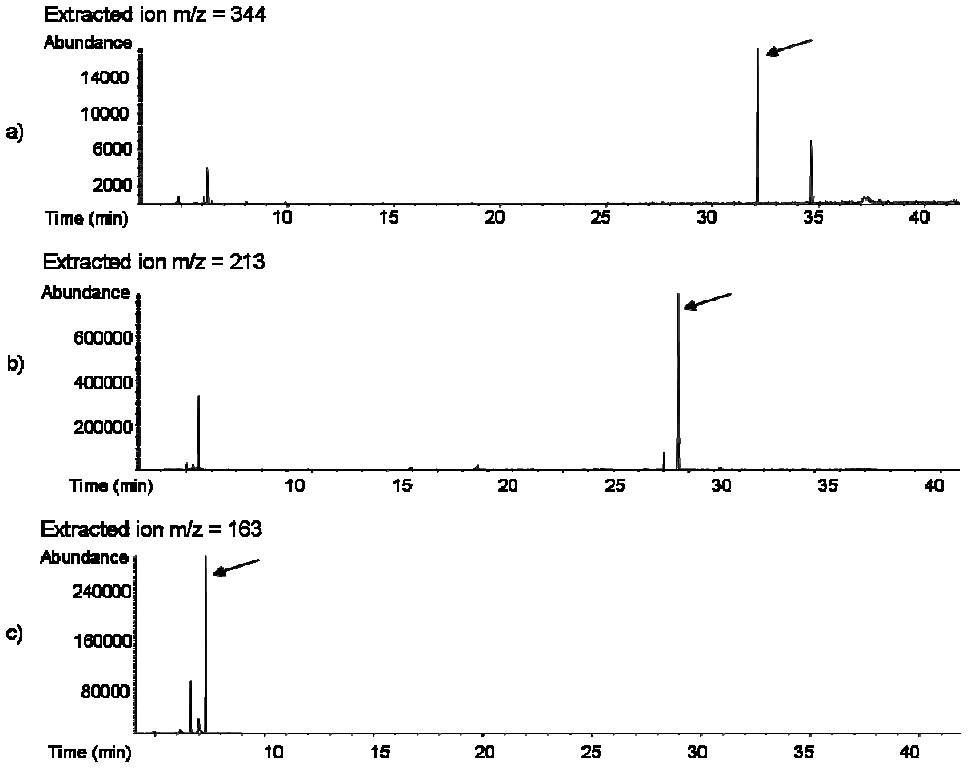


Figure III.18: Extracted ion chromatograms of the analysis of real-world water samples at m/z 344 for 17-β-(trimethylsilyl)estradiol acetate in Torino depuration plant (a), m/z 213 for bisphenol A diacetate in Torino depuration plant (b) and m/z 163 for propofol acetate in hospital effluent (c).

The derivatization step specific for carboxylic acids and amines allowed us to identify ketoprofen at a concentration of 320 ng/L in a process-water sample from Torino. The corresponding extracted ion chromatogram is given in **Figure III.19**.

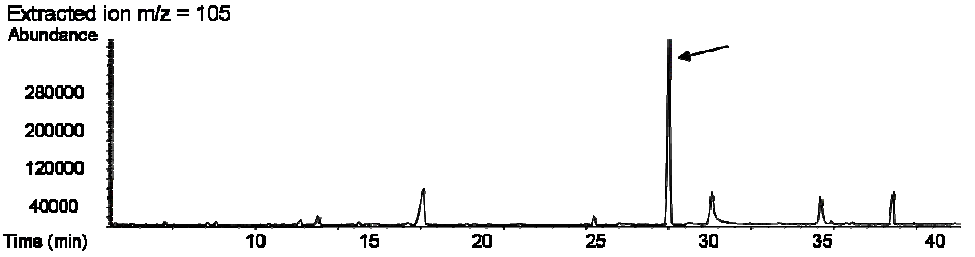


Figure III.19: Extraced ion chromatogram at m/z 105 for the detection of ketoprofen in water sample from Torino depuration plants.

The derivatization reaction using sodium tetraethylborate enabled the identification of tributyltinchloride in all samples from the Torino depuration plants. The highest amount detected was 486 ng/L (**Figure III.20**).

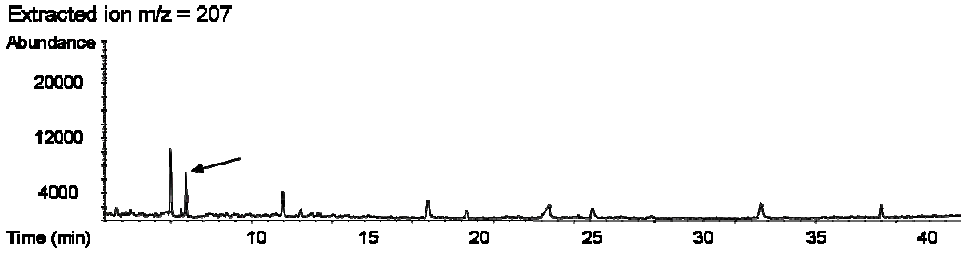


Figure III.20: Extracted ion chromatogram at m/z 207 for the detection of tributyltinchloride in water sample from Torino depuration plants.

The SBSE sampled underivatised aliquots of the water samples from Torino contained several phytosterols as silyl derivatives from in-*situ* BSTFA derivatization. β-sitosterol at concentrations as high as 130 ng/L were measured (**Figure III.21**).

Figure III.21: Extracted ion chromatograms at m/z 414 for the detection of β-sitosterol in water sample from Torino depuration plants.

In addition diethylphthalate, dibuthylphthalate and bis(2-ethylhexyl)phthalate were also identified. Quantitation of phthalates is a complex task because they are ubiquitous and it is extremely difficult to obtain a phthalate-free reference water. The problems related with their analysis have been discussed in depth elsewhere [50].

3.4 Conclusions

A multi-shot SBSE-TD-GC-MS method to analyse simultaneously different classes of EDCs and pharmaceuticals has been developed. Four different sample preparation procedures carried out in parallel on four aliquots of the same water sample are performed. The resulting stir bars are analysed by one thermal desorption process followed by GC-MS analysis. Three derivatisation reactions specific to phenolic compounds, amines and acids, and organometallic compounds, respectively, were applied to three sample aliquots, while compounds with a log $K_{o/w}$ compatible with PDMS and not requiring derivatisation were sampled in the fourth aliquot. In-tube silylation was carried out BSTFA.

The main advantages of the method include a) the possibility to screen simultaneously several EDCs and pharmaceuticals with different structures, b) with exception of the sample preparation step, the method is fully automated and c) only small volumes of samples are required while high recoveries and sensitivities can be achieved.

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CHAPTER IV
IMPROVING THE EXTRACTION OF POLAR ANALYTES

The extraction of polar analytes in aqueous samples is very difficult. In this chapter, two different strategies are evaluated in order to improve the extraction efficiency of these solutes.

In the first part of this chapter, a novel sorptive extraction technique is presented, namely silicone membrane sorptive extraction (SMSE). A PDMS tube is filled with an organic solvent and placed in the aqueous sample for extraction. Afterwards, the organic solvent in the PDMS tube is analysed by large volume injection GC-MS or LC-MS. First, the extraction was optimized for the determination of atrazine and its metabolites. Then, the performance of the presented method is evaluated in terms of linearity, repeatability, limits of detection and limits of quantification. The presented SMSE-GC-MS is able to screen for atrazine, desethylatrazine, desisopropylatrazine and desethyldesisopropylatrazine at low ng/L levels. The applicability of SMSE is evaluated for a complex mixture of EDCs and pharmaceuticals.

In the second part of this chapter, a new stir bar extraction material based on monoliths was prepared. The extraction capabilities of this material were evaluated for the static headspace analysis of coffee and compared to the conventional extraction with PDMS. .

1 Silicone membrane sorptive extraction*

1.1 Introduction

Triazine herbicides are one of the most important classes of chemical pollutants owing to their widespread use and toxicity. In the last two decades, atrazine has become the most frequently detected pesticide in surface and groundwater [1]. Atrazine is suspected as one of the endocrine disruptors in recent reports [2]. It has the capability to interrupt regular hormone function, causing birth defects, reproductive tumors, and weight loss in mother and embryo. In addition it can cause multiple types of cancers [3,4].

Once in the environment, atrazine is subjected to various biotic and abiotic degradation processes [5,6]. The main degradation products of atrazine (ATR) in water are the dealkylated chlorometabolites namely desethyldesisopropylatrazine (DDA), desisopropylatrazine (DIA) and mainly desethylatrazine (DEA). The structures of atrazine and its metabolites are shown in **Figure IV.1**.

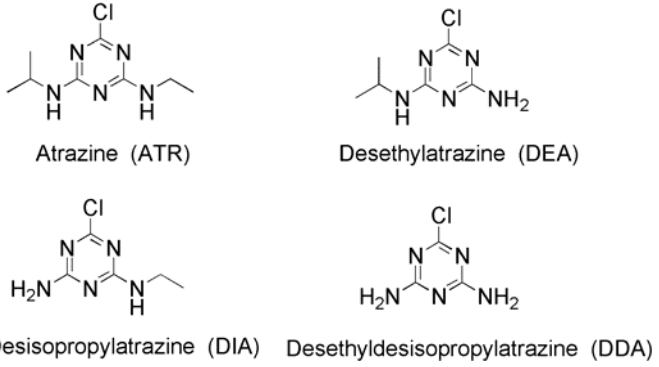


Figure IV.1: Structure of atrazine and its three chlorinated metabolites.

These degradation products, however, are as toxic, or even more so, than their parent compound [7]. For a complete understanding of the effect of the application of

* 'Determination of atrazine and its metabolites in aqueous samples using silicone membrane extraction (SMSE) followed by GC-MS and LC-MS',
E. Van Hoeck, E. Dumont, P. Sandra, submitted to Chromatographia

atrazine on the environment, it is therefore important to have an analytical method capable of quantifying atrazine and its three metabolites well below the maximum contaminant levels for pesticides (0.1 μg/L) mentioned in the EU water quality directive [8].

A variety of analytical methods are used to measure ATR using solid phase extraction (SPE) combined with either gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS). Many of these methods may also be used for the analysis of the monodealkylated metabolites DEA and DIA [9-16]. Due to the more polar nature of the completely dealkylated metabolite DDA (log $K_{o/w}$ = 0.32), its extraction from aqueous media is very challenging. As a result, only few methods for the determination of DDA in drinking water have been reported. One of the reported methods, developed by Lin and Yokley, utilizes graphite/cation exchange mixed mode SPE for the analysis of ATR, DEA, DIA and DDA [17]. Another method using C_{18} cation-exchange mixed mode SPE for analysis of ATR, DEA, DIA and DDA was later reported by Huang et al. [18]. SPE-chemical derivatization followed by GC-MS was applied by Carter et al. [19] and Panshin et al. [20] for the analysis of atrazine and the metabolites DEA, DIA and DDA. A liquid-liquid partitioning method followed by GC-MS was reported by Yokley and Cheung [21]. Jiang et al. described a procedure for the simultaneous determination of ATR and its three dealkylated metabolites using a combination of an Oasis MCX® SPE and carbon black SPE cartridge. The sample preparation was followed by GC-MS analysis [22]. Another method utilizing high-reslution mass spectrometry has been reported with SPE using C18-bonded [23] or a graphite-carbon cartridges [24]. A variety of other methods have been developed for triazine analysis at low concentrations using for example, immunosorbent SPE [25], and molecularly imprinted polymers [26]. In order to obtain maximum sensitivity for DDA all these methods utilises large sample volumes (up to 1L). Thus, there is a need for a method that will allow simultaneous determination of ATR, DEA, DIA and DDA that uses smaller sample volumes, more traditional analysis techniques and is able to screen for these contaminants at low-ppt (ng/L) level.

As is previously mentioned, the key to a sensitive method for trace analysis is an optimized pre-concentration procedure (Chapter II). However, application of SBSE in combination with derivatization was unsuccessful.

In this contribution, a novel sorptive extraction technique is presented, namely silicone membrane sorptive extraction (SMSE). A PDMS tube is filled with an organic solvent and placed in the aqueous sample for extraction. Afterwards, the organic solvent in the PDMS tube is analysed by large volume injection GC-MS or LC-MS. Several parameters that influence the extraction efficiency, the desorption efficiency and the analysis were tested. The performance of the GC-MS and LC-MS methods was evaluated in terms of linearity, repeatability and limits of detection and limits of quantitation. Finally, the usefulness of this technique is evaluated for a complex mixture of EDCs and pharmaceuticals.

1.2 Experimental

1.2.1 Chemicals

Neat certified standards of atrazine (purity 99.0%), atrazine-d_5 (ethyl-d_5) (98.4%) desethylatrazine (99.9), desisopropylatrazine (95.0%), desethyldesisopropylatrazine (97.8%), alachlor (99.9%), sulfamethoxazole and 3.4-dichloroaniline (99.3%) were purchased from Riedel de Haen (Seelze, Germany). Tripropyltinchloride (99.5%) was purchased from Dr. Ehrenstorfer (Augsburg, Germany). 4-t-butylphenol (99%), Propofol (97%), acetaminophen (98%) and bisphenol A (99%) were supplied by Aldrich (Bornem, Belgium). Estrone (99%), sulfamerarine (99%), ketoprofen, ibuprofen (98%), raniditidine hydrochloride, carbamazepine and 17-β-estradiol (98%) were purchased from Sigma (Bornem, Belgium). Caffeine (99%), sulfadimethoxine, metribuzin, trimethoprim and testosterone (99%) were supplied by Fluka (Bornem, Belgium).

The chemical structures of these EDCs and pharmaceuticals are given in **Figure IV.1** (p 106) and **Figure IV.12** (p 129).

Formic acid, water, methanol (MeOH) and acetonitrile (ACN) (all MS grade) were supplied by Biosolve (Valkenswaard, The Netherlands). Acetone, sodium chloride

(NaCl) and ethyl acetate (EA) (all HPLC grade) were purchased from Sigma-Aldrich (Bornem, Belgium). Stock solutions of each individual compound were prepared in acetone at a concentration of 1 mg/mL. The solutions were stored at 4°C and used to prepare the spiking solutions.

1.2.2 Sample preparation

Polydimethylsiloxane tubes (different length, 0.2 µm d_f) were kindly supplied by the Research Institute for Chromatography (Kortrijk, Belgium). Method development was done using 10 mL water samples spiked with 10 µL of a 500 ng/mL triazine standard solution and 10 µL of the atrazine-d_5 internal standard solution at the same concentration. This corresponds to 500 ng/L (ppt) in the water sample (or 5 ng per solute added to 10 mL sample). 0 to 4 g of NaCl was added to the aqueous sample to decrease the solubility of atrazine and its metabolites. The PDMS tube was filled with an organic solvent and closed at both ends. Different solvents (ethyl acetate, methanol and acetonitrile) were evaluated. Extraction was performed by placing the PDMS tube in the aqueous sample at room temperature while stirring at 500 rpm (Variomag Multipoint 6/15, H+P Labortechnik, München, Germany) for an extraction time ranging from 30 to 120 min. The set-up of SMSE is demonstrated in **Figure IV.2**. Afterwards the PDMS tube was removed from the aqueous solution and the organic solvent was analysed by GC-MS or LC-MS.

The performance of the method was evaluated by comparing the results obtained by the optimized SMSE method with those of the conventional SBSE method. Stir bars (10 mm x 0.5 mm containing 25 µL polydimethylsiloxane-PDMS coating) (Twisters) were obtained from Gerstel (Gerstel GmbH, Mülheim an der Ruhr, Germany). The SBSE extraction was done using 10 mL water samples spiked with the same concentrations as for SMSE. The water sample was saturated with 3 g NaCl. The extraction was performed by placing the Twister in the aqueous sample at room temperature while stirring at 500 rpm for 60 min. Afterwards the twister was analysed using TDS-GC-MS.

A more fundamental study was done using water samples of 10 mL, spiked with 10 µL of a 1/10/100 µg/mL standard solution of EDCs and pharmaceuticals. The

spiked water sample contained 4-t-butylphenol, 3.4-dichloroaniline, propofol, caffeine, estrone, tripropyltinchloride, metribuzin, bisphenol A, carbamazepine, alachlor, 17-β-estradiol and testosterone in a concentration of 1 ng/mL. Acetaminophen, ibuprofen and ranitidine HCl were present at a 10 ng/mL concentration. At last, sulfamethoxazole, sulfadimethoxine, ketoprofen, sulfamerazine and trimethoprim were at 100 ng/mL concentration. The extraction wass carried out at room temperature, with ethyl acetate in the PDMS tube, while stirring for 60 min at 500 rpm. After extraction, the ethyl acetate fraction was analysed using GC-MS(SIM). The corresponding PDMS tube was also analysed using thermal desorption followed by GC-MS(SIM).

1.2.3 Instrumental

Gas chromatography-mass spectrometry analyses were carried out on an Agilent 6890 gas chromatograph – 5975 mass spectroscopic detector combination (Agilent Technologies, Little Falls, DE, USA) equipped with a split/splitless inlet (S/SL) and a programmed temperature vaporization inlet (CIS-4, Gerstel GmbH, Mullheim, Germany). The temperature of the split/splitless inlet was set at 250°C. An injection volume of 1 µL was used and the injection was carried out in the splitless mode. When using the programmed temperature vaporization inlet, the injection volume was 10 µL. The initial temperature was set at 60°C for 0.3 min. Afterwards the injector was heated to 250°C (2 min) at 600°C/min. The vent pressure was initially set at 127 kPa and the split vent valve was open. After 0.25 min the valve was closed and at 1 min it was again opened. The purge flow was set at 50 mL/min.

The PDMS stir bars were analysed by TDS-GC-MS using a TDS-2 unit (Gerstel GmbH, Müllheim a/d Ruhr, Germany) mounted on the GC via the CIS-4 inlet. The stir bar was placed into a glass tube of 187 mm L, 6 mm OD and 4 mm ID. Splitless thermal desorption was performed by programming the TDS from 35°C (1 min) to 300°C (5 min) at a rate of 60°C/min with a helium flow rate of 100 mL/min. The analytes were cryo-focussed in the CIS-4 inlet at -150°C using liquid nitrogen. Splitless injection was performed by ramping the CIS-4 from -150°C (0.10 min) to 300°C (5 min) at a rate of 10°C/s.

GC-MS analysis were carried out on both a DB-WAX and a DB-17MS column. The first series of analyses were carried out on a DB-WAX fused silica capillary column (polyethylene glycol) of 10 m L, 0.25 mm ID and a phase thickness of 0.25 µm (Agilent Technologies, Folsom CA, USA). The temperature was programmed from 60°C (0.3 min) to 250°C at a rate of 25°C/min. This temperature was then maintained for 5 min.

In order to obtain better sensitivities, large volume injection was carried out on a semi-polar DB-17MS fused silica capillary column (50% diphenyl-dimethylpolysiloxane) of 30 m L, 0.25 mm ID and a phase thickness of 0.25 µm (Agilent Technologies, Folsom, CA, USA). The initial temperature was set at 50°C (1 min). Afterwards, the column was heated to 300°C (1 min) at 25°C/min. Helium was used as carrier gas on both columns at a constant flow rate of 2.9 mL/min for the DB-WAX column and at 1.5 mL/min for the DB-17MS column.

For the fundamental study, the analyses were carried out on a DB-17MS column in combination with a splitless injection of 1 µL. The following temperature program was used: initial temperature was set at 70°C and then heated to 300°C (17 min) at 10°C/min. Helium was used as carrier gas at a constant pressure of 120 kPa.

Detection was carried out in the selected ion monitoring (SIM) mode. The transfer line, ion source and quadrupole analyser temperatures were set at 280°C, 230°C and 150°C respectively, and a solvent delay of 4 min was used. Electron ionisation mass spectra were recorded at 70eV electron energy with an ionisation current of 34.6 µA. Three characteristic ions for each compound were selected namely a target ion for quantification and two qualifier ions. The SIM groups are listed in **Table IV.1** for the atrazine study and in **Table IV.5** for the more fundamental study. The dwell time was set at 80 ms. Data acquisition, instrument control and data analysis were performed by ChemStation software (G1701CA, version C.00.00, Agilent Technologies).

Table IV.1: Octanol-water partition coefficients, retention times, selected SIM ions and corresponding SIM groups for the triazines studied by GC-MS on both the DB-17 MS and the DB-WAX column and by LC-MS (see further).

	Log $K_{O/W}$*	SIM ions[$]	GC-MS				LC-MS	
			DB-WAX		DB-17MS			
			RT (min)	SIM group	RT (min)	SIM group	RT (min)	SIM group
DDA	0.32	68, 110, *145*	10.54	4	8.85	1	7.86	1
DIA	1.15	145, 158, *173*	9.13	3	9.13	2	11.31	2
DEA	1.51	*172*, 174, 187	8.77	2	9.05	2	12.47	3
ATR-d_5		58, *205*, 220	7.99	1	9.26	3	14.30	4
ATR	2.61	58, *200*, 215	8.01	1	9.28	3	14.32	4

* Octanol/water coefficients are obtained by the software program SRC-KOWWIN

[$]Target ions in *ITALIC*

LC-MS analyses were carried out on an Alliance 2690 LC system equipped with an on-line degasser and autosampler. The sample was separated on a Luna-C18 column (150 mm L x 2.1 mm ID, 5 µm d_p) (Phenomenex, Torrance CA, USA). The column was thermostated at 20°C. The mobile phase consisted of methanol and water containing 0.1% formic acid. A linear gradient program was used from 0% methanol to 100% methanol in 10 min. This mobile phase composition was then maintained for 10 min. Afterwards, the mobile phase composition returns to the initial conditions. The flow rate was 0.2 mL/min and the injection volume was between 10 and 100 µL depending on the organic solvent used for the sample preparation.

Detection was carried out using a Quattro Micro system equipped with a Z-spray electrospray ionization source (Micromass, Manchester, UK). The mass spectrometer was used in the positive mode. The capillary voltage was set at 3 kV and the cone voltage at 30 V. The source temperature and desolvatation temperature were at 120°C and 350°C, respectively. A cone gas flow of 50 L/h and a desolvation gas flow of 350 L/h were applied.

Mass spectra were recorded in the SIM mode. One characteristic ion for each compound was selected. The SIM groups are also listed in **Table IV.1**. The dwell time

was 100 ms. Data acquisition, instrument control and data analysis were performed by Masslynx software (version 4.0, Micromass).

1.3 Results and discussion

1.3.1 Principle of silicone membrane sorptive extraction

The experimental set-up for silicone membrane sorptive extraction (SMSE) is presented in **Figure IV.2**.

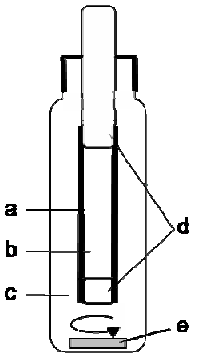


Figure IV.2: Device for SMSE: A PDMS tube (a) is filled with organic solvent (b), closed at both ends (d) and placed in an aqueous solution (c). During the extraction, the sample is stirred with a glass stir bar (e).

A PDMS tube with a length of 4 cm is filled with an organic solvent, closed at both ends and placed in the aqueous sample. First, the analytes are absorbed in the PDMS layer. Due to the presence of an organic solvent inside the PDMS tube, the absorbed analytes are further extracted in this solvent, leading to higher extraction efficiencies. This is the major advantage of this novel sample preparation technique.

The theoretical enlightenment of this type of extraction is very complex due to the different equilibrations that take place. Furthermore, the properties of PDMS change due to the presence of the organic solvent, which also partitions in the PDMS phase. Therefore, the enlightenment of the theory behind this type of extraction is beyond the scope of this work and will not be discussed.

1.3.1.1 Optimization of the SMSE procedure

Main variables in SMSE are the length of the PDMS tube and the nature and quantity of the organic solvent used. Note that the sample volume for these studies was fixed at 10 mL.

PDMS tubes with lengths varying from 3 to 5 cm were applied for the analysis of a water sample (10 mL) spiked at 500 ng/L and saturated with 3 g NaCl. The extraction was carried out at room temperature, with ethyl acetate in the PDMS tube, while stirring for 60 min at 500 rpm. After extraction, 1 µL of the ethyl acetate fraction was analysed using GC-MS in the split/splitless inlet and on the DB-WAX column. The results, expressed as relative peak areas normalized to 4 cm PDMS, are shown in **Figure IV.3.**

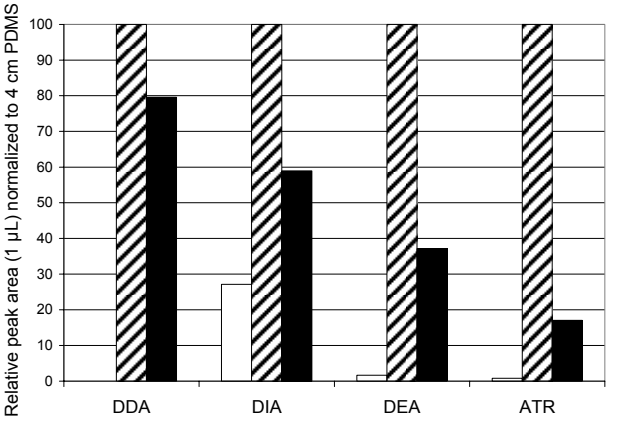


Figure IV.3: Influence of the length of the PDMS tube on the recovery of the triazines. Analysis is performed by SMSE(EA)-GC (1 µL, S/SL, DB-WAX))-MS(SIM): 3 cm PDMS (□), 4 cm PDMS (▨) and 5 cm PDMS (■).

As can be seen from this figure, the best results were obtained when 4 cm of PDMS was used and consequently 4 cm PDMS was used for further experiments.

In the past, low recoveries for polar compounds such triazines have been observed when working with SPME and SBSE due to the very good solubility of these compounds in aqueous samples [27]. The metabolites of atrazine are very polar, as is reflected by their low log $K_{o/w}$ values in **Table IV.1**. As a result, low recoveries are expected. A series of experiments was, therefore, performed using salt addition to

decrease the solubility of atrazine and its metabolites in aqueous samples. Different amounts of sodium chloride (NaCl) were added to the sample (10 mL spiked at 500 ng/L), varying from 0 to 3 g. It should be noted that with 3 g NaCl in 10 mL water, the saturation level is reached. The extraction was carried out using a PDMS tube with a length of 4 cm and 150 µL ethyl acetate. The aqueous solution was stirred at room temperature for 60 min at 500 rpm. Afterwards, the ethyl acetate fraction was analysed by GC-MS using split/splitless injection on the DB-WAX column.

When less than 3 g NaCl was added to the aqueous sample, at the end of the extraction the ethyl acetate had completely disappeared. This can be explained by the high solubility of ethyl acetate in water. At room temperature, 600 µL ethyl acetate can dissolve in 10 mL water. This corresponds with a volume percentage of 6%. Since the PDMS tube contains only 150 µL, all the ethyl acetate can dissolve in the aqueous solution with a volume of 10 mL. By adding salt to the aqueous sample, the solubility of ethyl acetate is decreased. As a consequence, when 3 g NaCl is added to the aqueous sample, the volume inside the PDMS tube at the end of the extraction is reduced to 10 µL. Therefore, 3 g NaCl was selected for further experiments.

Next, the influence of the organic solvent in the PDMS tube was evaluated using a constant sample volume of 10 mL with 3 g NaCl. A PDMS tube with a length of 4 cm was filled with 150 µL acetonitrile, ethyl acetate and methanol and placed in the aqueous solution which was stirred at room temperature for 60 min at 500 rpm. The results, expressed as relative recoveries normalised to ethyl acetate are shown in **Figure IV.4** .

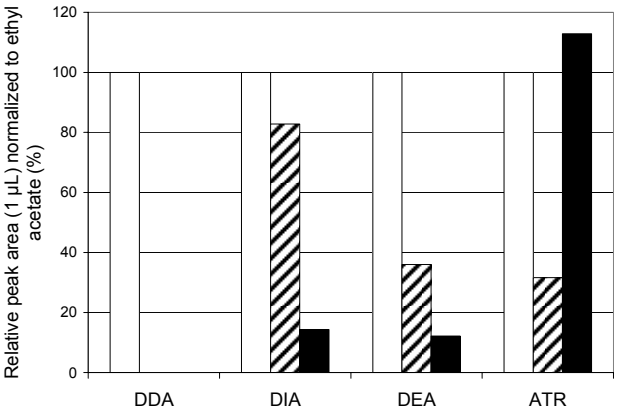


Figure IV.4: Influence of the organic solvent in the PDMS tube on the recovery of the triazines by SMSE-GC (1 µL, S/SL, DB-WAX)-MS(SIM):Ethyl acetate (□), Acetonitrile (▨) and methanol (■).

From this figure, it can be seen that DDA is only extracted when using ethyl acetate. In addition, ethyl acetate delivers the best recovery for all the metabolites. As a consequence, ethyl acetate was selected for further experiments.

Finally, the influence of the extraction time was evaluated using a constant sample volume of 10 mL saturated with 3 g NaCl and a PDMS tube of 4 cm filled with 150 µL ethyl acetate. Different extraction times, ranging from 15 to 120 min were evaluated. The recovery versus extraction time plots, presented in **Figure IV.5** , showed that equilibrium conditions were reached after ca. 30 min.

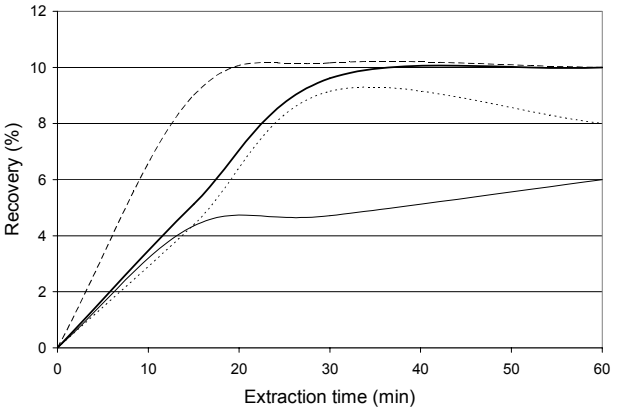


Figure IV.5: Influence of the extraction time on the recovery of the triazines by SMSE-GC (1 µL, S/SL, DB-WAX)-MS(SIM): DDA (——), DIA (·····), DEA (- - - -) and ATR (▬▬).

As previously mentioned, during the extraction, ethyl acetate moves through the PDMS tube and dissolves in the aqueous sample. The fraction of ethyl acetate that is dissolved in the aqueous phase depends on the extraction time. When the sample is stirred for 15 min, the volume ethyl acetate left in the tube is 60 µL, while after 60 min only 10 µL ethyl acetate is left in the PDMS tube. After 2 h, all of the ethyl acetate is dissolved in the aqueous phase. Due to the decreasing volume of ethyl acetate versus the extraction time, higher sensitivities can be obtained using a longer extraction time, since the injection volume is independent of the extraction time. This is illustrated by plotting the areas for the injection of 10 µL in function of the retention time as is shown in **Figure IV.6**.

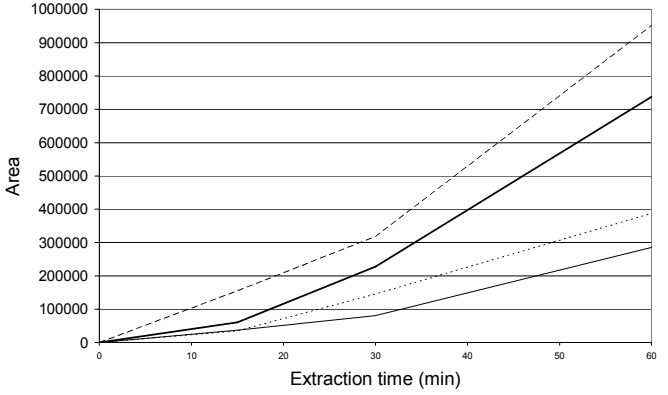


Figure IV.6: Influence of the extraction time on the area of the triazines by SMSE-GC (10 µL,S/SL, DB-WAX)-MS(SIM): DDA (——), DIA (·····), DEA (- - - -) and ATR (▬▬).

From this figure it can be concluded that an extraction time of 60 min is giving the maximum sensitivity. This was used for further experiments.

1.3.1.2 Performance of the SMSE method

The recoveries obtained by SMSE under the selected conditions, i.e. 10 mL sample saturated with 3 g NaCl, 4 cm PDMS with 150 µL ethyl acetate and stirred at room temperature for 60 min at 500 rpm followed by GC (10 µL, LVI, DB-17MS)-MS(SIM) were measured by comparison of the peak area of the solutes for a 500 ng/L spiked water sample with those obtained by direct liquid injection of 10 µL of 500 µg/L triazine mixture. The theoretical recovery for PDMS can be calculated using the equation on p 44. The theoretical recoveries for SBSE are enlisted in **Table IV.2** together with the experimental recovery in the EA extract after SMSE.

Table IV.2: Theoretical recovery for PDMS after SBSE and experimental recovery of the EA extract after SMSE from the analysis by GC (10 µL, LVI, DB-17MS)-MS(SIM).

	Recovery (%)	
	Theoretical	**Experimental**
ATR	49	10
DEA	7	10
DIA	3	8
DDA	0	6

From this table it can be seen that for the very polar DDA, the recovery obtained after SMSE with ethyl acetate is much higher than the theoretical recovery when SBSE is used. This is probably caused by the presence of ethyl acetate inside the PDMS tube. The polar nature of DDA leads to low affinity towards the PDMS phase. Once it is absorbed in the PDMS layer, it is thus immediately further transferred in the ethyl acetate leading to a higher recovery in the EA extract and pushing re-equilibration. On the contrary, atrazine which is less polar will show more affinity for the PDMS phase, leading to lower recoveries in the ethyl acetate fraction in comparison with the theoretical recovery after SBSE.

An in depth explanation of these results is very complicated, due to the different equilibrations that take place. A first equilibrium occurs between the aqueous and PDMS phase followed by equilibrium between PDMS and ethyl acetate. These two equilibriums continuously influence each other. Furthermore, the properties of PDMS change due to the presence of EA, which also partitions in the PDMS phase. As a consequence, the theoretical enlightenment of this type of extraction is very complex and beyond the scope of this work.

The performance of the optimized SMSE method was evaluated with several experiments. First, the PDMS tube after extraction in presence of EA was analyzed by TDS-GC(DB-17MS)-MS(SIM). Since the triazines are extracted in the PDMS phase, before their transfer in the organic solvent, recoveries in PDMS can be higher than those in the EA extract, depending on the equilibrium between PDMS and EA. The influence of the presence of ethyl acetate was investigated by performing the same SMSE procedure, but without organic solvent. Finally, the optimized SMSE procedure was compared with conventional SBSE-TDS-GC(DB-17MS)-MS(SIM). The results of all these experiments, expressed as relative recoveries normalized to the EA extract, are depicted in **Figure IV.7**.

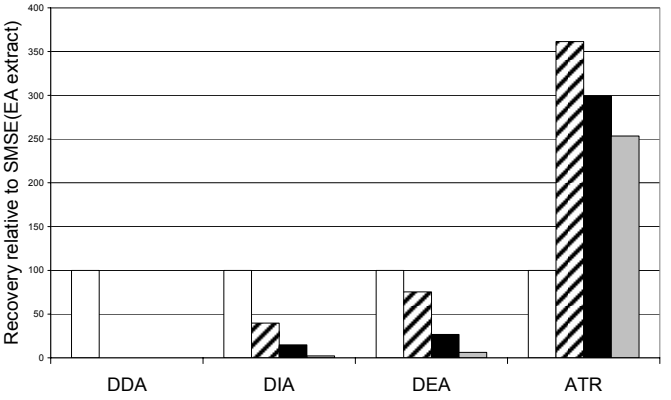


Figure IV.7: Influence of the presence of EA in the PDMS tube on the recovery: SMSE: EA extract (□),SMSE: PDMS of the EA extractl (▨), SMSE: PDMS without organic solvent (■), SBSE (▩).

As can be seen from this figure, the polar metabolite DDA is only present in the EA extract. In addition, the recovery in the PDMS tube after extraction with ethyl acetate increases with decreasing polarity. Furthermore, due to the presence of ethyl acetate in the PDMS tube, the obtained recoveries are higher compared to the empty PDMS tube. An explanation is that the SMSE extraction consists of two consecutive extractions. First, the compounds are extracted by the PDMS and then they are further transferred in the organic solvent. As a consequence, the equilibrium between PDMS and water is disturbed, leading to the extraction of more analytes by PDMS. Finally, the SBSE extraction is less efficient than extraction by SMSE since the amount of PDMS is much lower on the twister (25 µL) than on the PDMS tube (4 cm corresponds to 116 µL PDMS).

Analysis with GC-MS

The optimization of the sample preparation was carried out using GC-MS on a polar DB-WAX column and an injection volume of 1 µL. **Figure IV.7** shows the ion chromatograms from the GC (1µL, S/SL, DB-WAX)-MS analysis of a 10 mL water sample spiked at the 500 ng/L level under optimized conditions. The unlabeled peaks present at m/e = 145 originate from column bleeding due to the use of the column at its maximum allowable temperature.

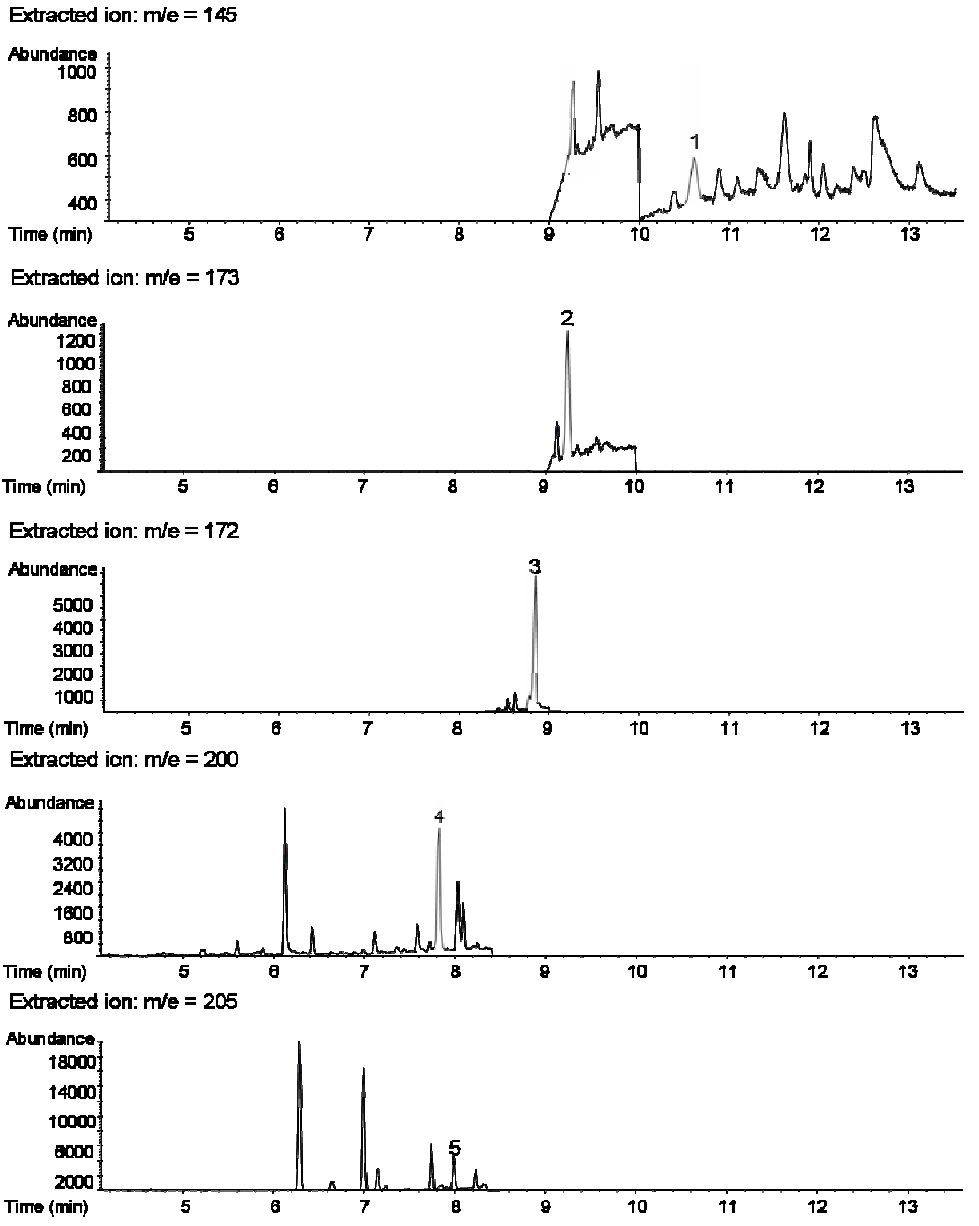
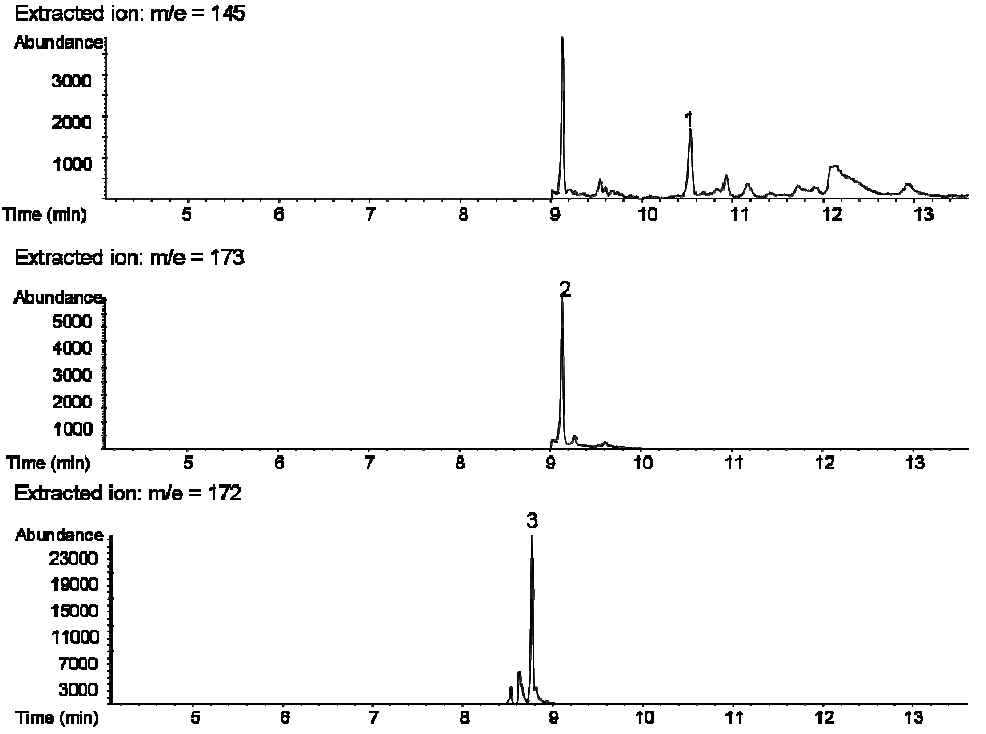


Figure IV.8: Extracted ion chromatograms of the triazines from an assay on a 10 mL water sample spiked at 500 ng/L (ppt) level by SMSE-GC (1 µL, S/SL, DB-WAX)-MS(SIM). The triazines in this chromatogram are DDA (1), DIA (2), DEA (3), ATR (4), ATR-d_5(5).

When the sample preparation is carried out under optimized conditions, the final volume of ethyl acetate is 10 µL. This complete extract can be analyzed using large volume injection. Unfortunately, injection of 10 µL ethyl acetate on the polar DB-WAX column causes severe peak distortion due to the incompatibility of the polyethylene glycol stationary phase with ethyl acetate. As a consequence, the injection volume had to be reduced. Different injection volumes were evaluated and 5µL ethyl acetate was the highest amount that could be injected without peak distortion. Therefore, 5 µL was selected for further experiments. The ion chromatograms from the GC (LVI-DB-WAX)-MS analysis (5 µL injection of the obtained extract) of a 10 mL water sample spiked at the 500 ng/L under optimized conditions are given in **Figure IV.9.**



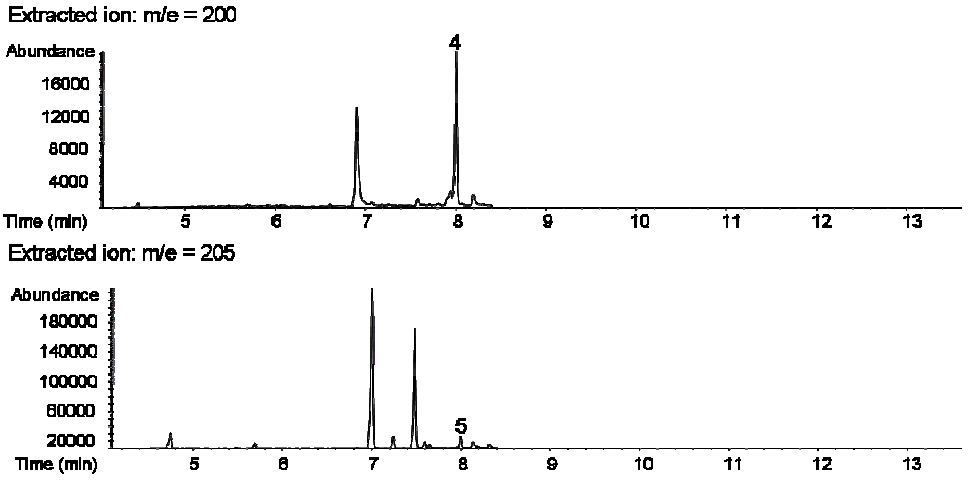


Figure IV.9: Extracted ion chromatograms of the triazines from an assay on a 10 mL water sample spiked at 500 ng/L (ppt) level by SMSE-GC (5 µL, LVI, DB-WAX)-MS(SIM). The triazines in this chromatogram are DDA (1), DIA (2), DEA (3), ATR (4), ATR-d_5(5).

Since the peak distortion is caused by incompatibility of the polar polyethylene glycol stationary phase and ethyl acetate, this problem could also be solved by changing to another stationary phase. The most convenient apolar stationary phase is HP-5MS (5% diphenyl, 95% dimethylsiloxane). Due to the polar nature of the metabolites of atrazine, peak distortion occurred due to incompatibility of the analytes and the stationary phase. Therefore, a semi-polar column was chosen namely DB-17MS (50% diphenyl-dimethylpolysiloxane). Different injection volumes of ethyl acetate were evaluated on this column and it was concluded that an injection volume of 10 µL could be used. As a result, the EA extract could be completely analyzed. The different ion chromatograms from the GC (LVI-DB-17MS)-MS analysis (10 µL injection volume) of a 10 mL water sample spiked at the 500 ng/L under optimized conditions are shown in **Figure IV.10**.

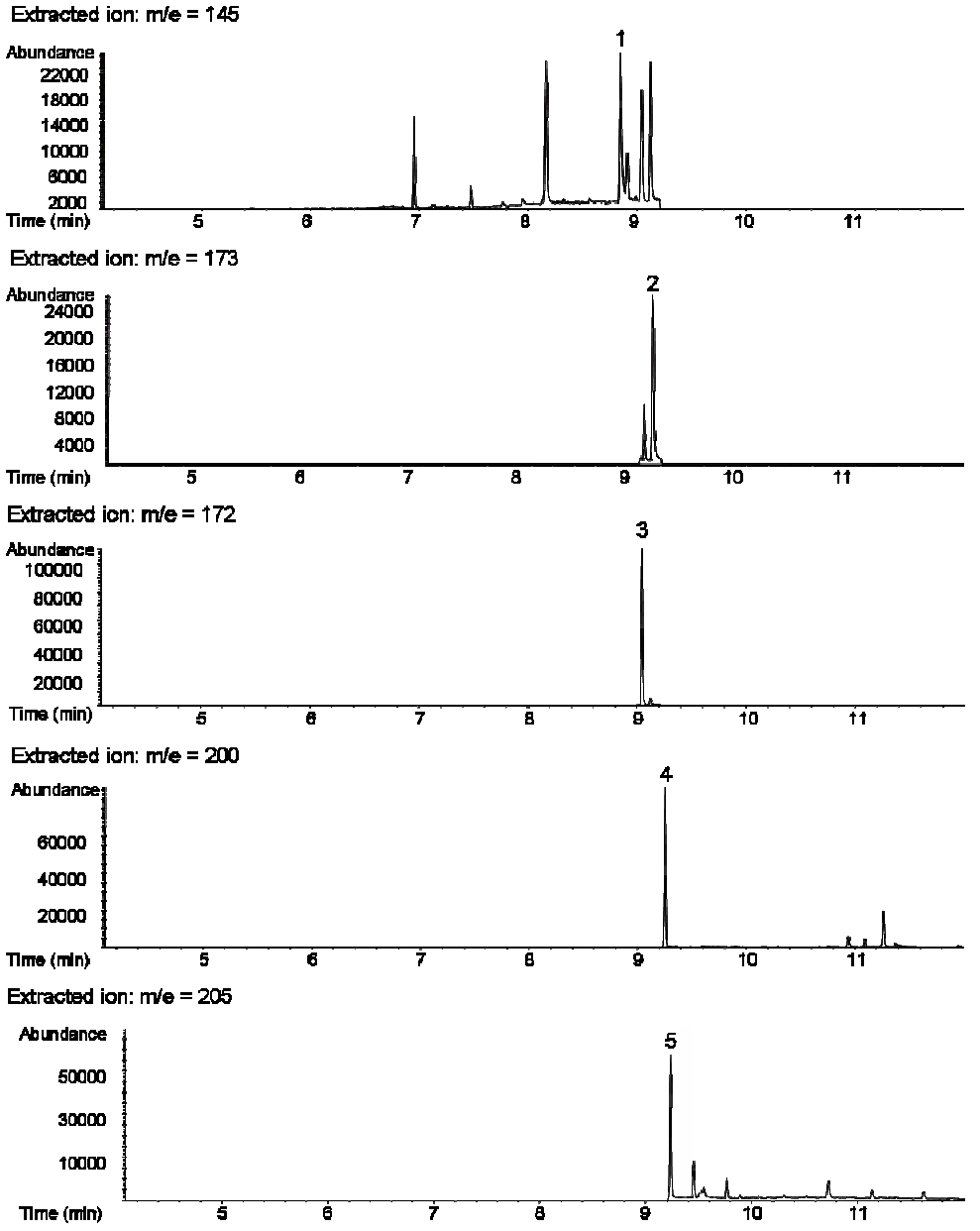


Figure IV.10: Extracted ion chromatograms of the triazines from an assay on a 10 mL water sample spiked at 500 ng/L (ppt) level by SMSE-GC (10 µL, LVI, DB-17MS)-MS(SIM). The triazines in this chromatogram are DDA (1), DIA (2), DEA (3), ATR (4), ATR-d_5(5).

The performance of the optimized method was studied for 10 µL analysis on the semi-polar DB-17MS column. The linearity was evaluated by extracting spiked water samples at 7 concentration levels (10, 50, 100, 250, 500 750 and 1000 ng/L). The calibration curves were obtained by plotting the peak ratios (triazine/atrazine-d_5) versus the concentration. All investigated triazines showed good linearity (R^2 values between 0.9936 and 0.9989) in the investigated range (**Table IV.3**).

Table IV.3: Performance of the SMSE-GC-MS under optimized conditions on both the DB-WAX and the DB-17MS column.

	R^2	Repeatability (%)	Column		
			DB-WAX	DB-17MS	
			LOD (ng/L)	LOD (ng/L)	LOQ (ng/L)
ATR	0.999	1	1.84	0.47	1.52
DEA	0.998	3	4.66	1.98	4.30
DIA	0.994	8	7.38	1.25	4.86
DDA	0.994	6	20.4	6.07	20.25

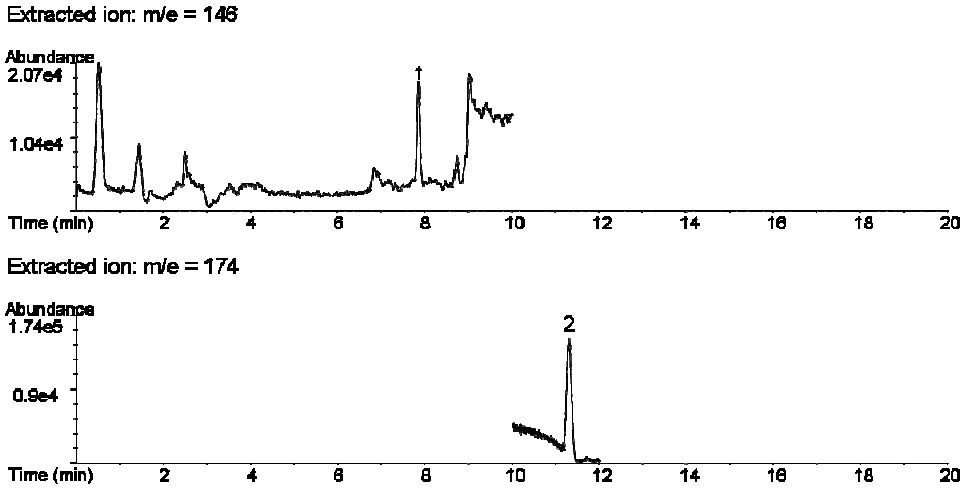
The repeatability was evaluated by analyzing six water samples spiked at 100 ng/L (n = 6). The relative standard deviations are included in **Table IV.3**. The RSDs were in the range of 1 to 8%. The sensitivity, expressed by the LOD values at S/N 3 and the LOQ values at S/N 10, are included in **Table IV.3** for both the optimized analysis on the DB-WAX column (5 µL injection volume) and on the DB-17MS column (10 µL injection volume). The LOD values are lower for the analysis on the DB-17MS column in comparison with the analysis on the DB-WAX column. This is caused by several factors. Firstly, the injection volume on the DB-WAX column is only 5 µL in comparison with 10 µL for the analysis on the DB-17MS column. Secondly, the DB-WAX column is less robust leading to bleeding of the column and consequently to higher detection limits.

The LOD values for the DB-17MS column are below 2 ng/L (ppt) for all solutes with exception of the most polar desethyldesisopropylatrazine (ca. 6 ng/L), showing excellent sensitivity of SMSE.

Analysis with LC-MS

The same sample preparation procedure, i.e. 10 mL water sample saturated with 3 g NaCl, 4 cm PDMS with 150 µL EA and extraction for 60 min at 500 rpm and room temperature, was also used in combination with LC-MS. Direct injection of the extract on reversed phase LC, leads to severe peak distortion, caused by solvent incompatibility between the organic solvent used for the extraction and the initial mobile phase composition. Therefore, the organic extract needs to be diluted prior to injection. In order to reach maximum sensitivity, different injection volumes and different organic solvent/water ratios were evaluated off-line. It was concluded that maximum sensitivity for both atrazine and its metabolites was obtained with 100 µL 6% ethyl acetate. The same experiments were carried out with acetonitrile (ACN) and methanol (MeOH). For these two solvents, maximum sensitivity was achieved with 10 µL 10% ACN in water and 50 µL 50% MeOH in water.

The different ion chromatograms from the LC (100 µL 6% EA)-MS(SIM) analysis of a 10 mL water sample spiked at the 500 ng/L under optimized conditions are shown in **Figure IV.11.**



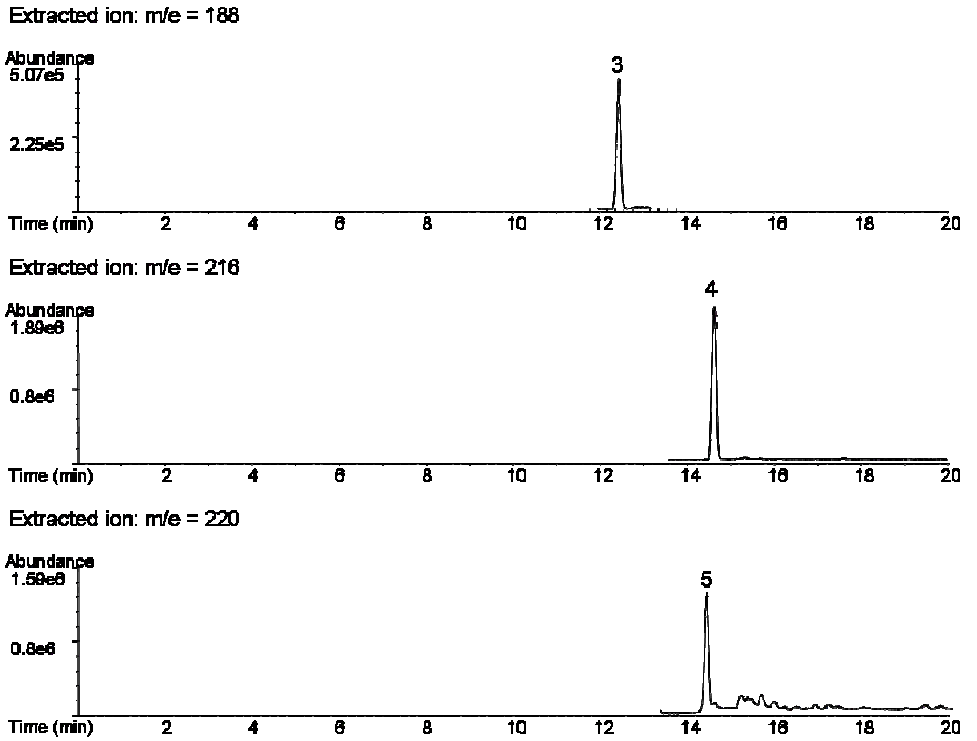


Figure IV.11: Extracted ion chromatograms of the triazines from an assay on a 10 mL water sample spiked at 500 ng/L (ppt) level by SMSE-LC (100 µL, 6% EA in water)-MS(SIM). The triazines are DDA (1), DIA (2), DEA (3), ATR (4), ATR-d_5(5).

The performance of SMSE-LC-MS was also evaluated in terms of linearity, repeatability and limits of detection. The linearity was evaluated by extracting spiked water samples at 7 concentration levels (10, 50, 100, 250, 500 750 and 1000 ng/L) under the selected conditions, i.e. 10 mL sample saturated with 3 g NaCl, 4 cm PDMS with 150 µL ethyl acetate and stirred at room temperature for 60 min at 500 rpm followed by dilution to 6% ethyl acetate in water and analysis using liquid chromatography-mass spectrometry. The calibration curves were obtained by plotting the peak ratios (triazine/atrazine-d_5) versus the concentration. All investigated triazines showed good linearity (R^2 values between 0.990 and 0.997) in the investigated range (**Table IV.4**).

Table IV.4: Performance of the SMSE-LC-MS under optimized conditions.

	R^2	Repeatability (%)	LOD (ng/L)	LOQ (ng/L)
DDA	0.996	12	40.9	130
DIA	0.992	14	7.9	26
DEA	0.997	11	1.8	6.5
ATR	0.990	3	1.0	3.2

The repeatability was evaluated by analyzing six water samples spiked at 500 ng/L (n = 6). The relative standard deviations are included in **Table IV.4**. The RSDs were in the range of 3 and 14%. The sensitivity, expressed by the LOD values at S/N 3 and the LOQ values at S/N 10, are included in **Table IV.4**. The LOD values are below 8 ng/L (ppt) for all solutes, except for DDA (130 ng/L), showing acceptable sensitivity of the method. Compared to GC-MS, reduced sensitivity is caused by the dilution effect.

1.3.2 Fundamental study on SMSE

From the analyses of atrazine and its metabolites, it could be concluded that silicone membrane sorptive extraction is beneficial for polar compounds. In order to further investigate the applicability of this new sample preparation technique, a complex mixture of EDCs and pharmaceuticals, with a wide variety in log $K_{o/w}$ values (**Table IV.5**), was analyzed using SMSE.

The EDCs and pharmaceuticals used for this study are shown in **Figure IV.12**.

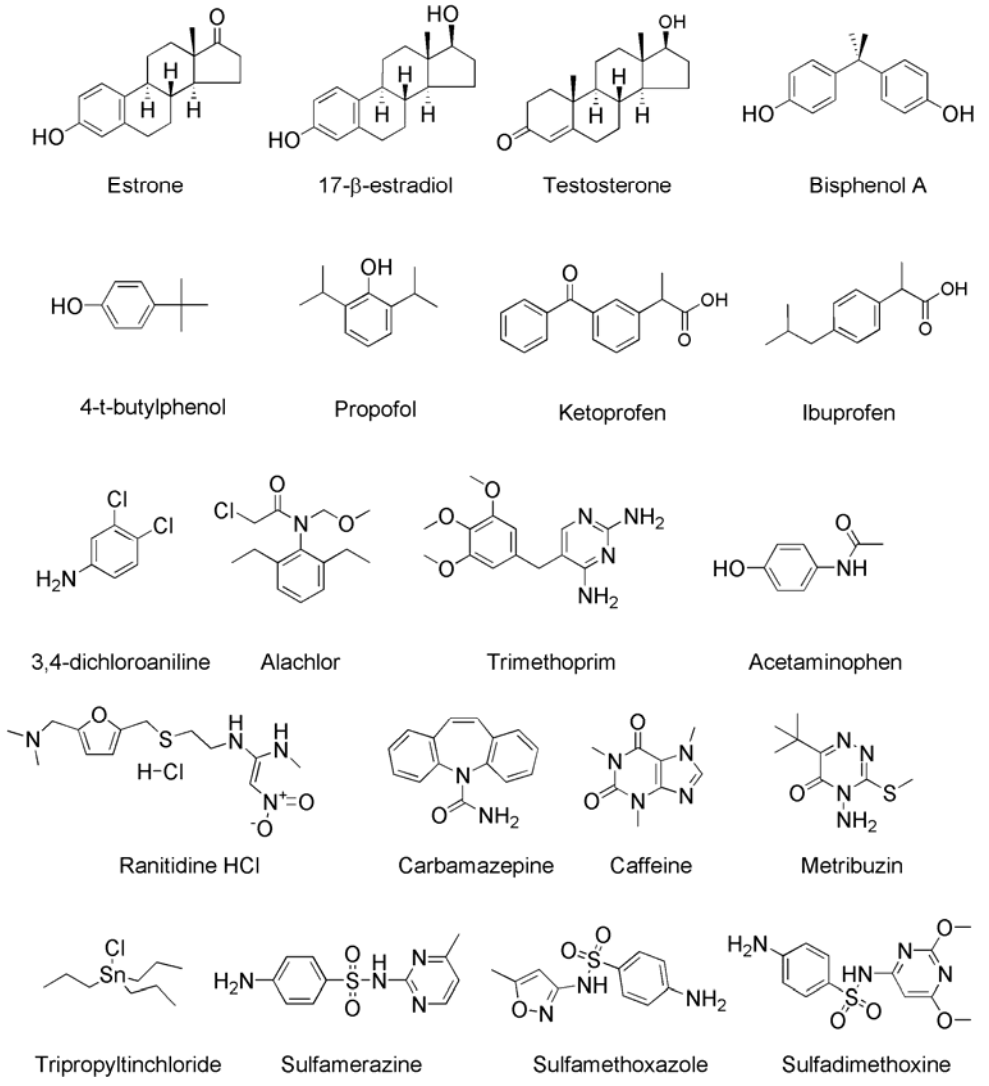


Figure IV.12: Structures of the EDCs and pharmaceuticals used for the fundamental study.

The log $K_{o/w}$ values vary between 0 and 4 (**Table IV.5**). Analytes with higher log $K_{o/w}$ values are not evaluated since these apolar solutes are already very well extracted with conventional SBSE. Furthermore, it has been shown by Beltran et al, that salt addition has a negative influence on the extraction efficiency of very apolar

compounds [28]. Here, the focus is placed on polar compounds and therefore, an aqueous solution saturated with salt was used.

The optimized sample procedure for the determination of atrazine and metabolites was used for the analysis of this complex mixture of EDCs and pharmaceuticals. Afterwards, both the PDMS tube and the ethyl acetate extract were analyzed by GC (DB-17MS)-MS(SIM). While the PDMS tube was thermally desorbed, 1 µL of the EA extract was analyzed in the split/splitless inlet of the GC. The retention times, SIM ions and corresponding SIM groups for the EDCs and pharmaceuticals, are given in **Table IV.5**.

Table IV.5: Octanol-water partition coefficients, retention times, selected SIM ions and corresponding SIM groups of the EDCs and pharmaceuticals.

Reference EDCs and pharmaceuticals	Log $K_{o/w}$*	Retention time (min)	Target ion$ and Qualifiers	SIM group
Caffeine	0.16	17.63	67, 109, *194*	6
Sulfamerazine	0.21	27.66	92, 199, *200*	13
Acetaminophen	0.27	15.76	43, *109*, 151	4
Ranitidine HCl	0.29	19.63	*137*, 297, 314	8
Sulfamethoxazole	0.48	20.46	*92*, 108, 156	9
Trimethoprim	0.73	26.03	259, 275, *290*	12
Sulfadimethoxine	1.17	32.71	65, 92, *246*	14
Metribuzin	1.49	17.43	144, *198*, 214	6
Carbamazepine	2.25	23.4	165, *193*, 236	10
3,4-dichloroaniline	2.37	11.55	99, *161*, 163	2
Ketoprofen	3	18.7	77, *105*, 254	7
Tripropyltinchloride	3.23	8.38	199, 239, *241*	1
Testosterone	3.27	25.32	*124*, 147, 288	11
Alachlor	3.37	16.59	*45*, 160, 188	5
4-t-butylphenol	3.42	8.21	107, *135*, 150	1
Estrone	3.43	25.54	185, 213, *270*	11
Propofol	3.57	8.98	117, *163*, 178	1
Bisphenol A	3.64	20.67	119, *213*, 228	9
Ibuprofen	3.79	12.56	161, *163*, 206	3

Reference EDCs and pharmaceuticals	Log $K_{o/w}$*	Retention time (min)	Target ion$ and Qualifiers	SIM group
17-β-estradiol	3.94	25.41	160, 213, *272*	11

* Octanol/water coefficients are obtained by the software program SRC-KOWWIN
$Target ions in *ITALIC*

The results of these analyses are presented as recovery in function of log $K_{o/w}$ in **Figure IV.13.**

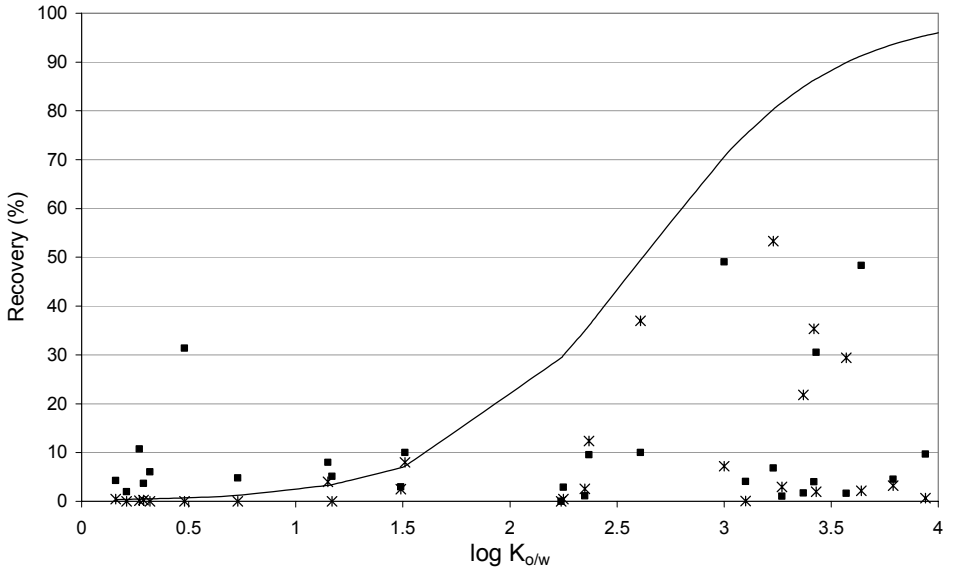


Figure IV.13: Recovery of the analytes in the ethyl acetate extract and the PDMS tube in function of log $K_{o/w}$: ethyl acetate extract (■), PDMS of SMSE with EA (✳) and the theoretical recovery for PDMS of SBSE (——).

From this graph, it can be seen that SMSE is only beneficial for very polar compounds (log $K_{o/w}$ < 2) since the recovery in the ethyl acetate extract is always higher than that of the PDMS tube and even higher than the theoretical recovery for SBSE. When less polar analytes are determined, the beneficial effect of ethyl acetate is disappearing as these compounds already show high affinity for PDMS.

In conclusion, this method should only be used for the analysis of very polar compounds.

1.4 Conclusion

A novel sorptive extraction technique is presented, namely silicone membrane sorptive extraction. A method was developed and optimized for the determination of atrazine and its metabolites desethylatrazine, desisopropylatrazine and desethyldesisopropylatrazine in aqueous samples using this type of sample preparation. Ethyl acetate was selected as organic solvent in the PDMS tube. Due to its presence, the extraction efficiency is significantly increased, leading to a higher sensitivity. The limited solubility of ethyl acetate in water causes the need for dilution of the extract with water before LC-MS analysis. GC-MS in combination with large volume injection is therefore more preferable, leading to higher sensitivities. A DB-17MS column was preferred over a DB-WAX column, since the latter showed peak distortion of the analytes in combination with large volume injection. The SMSE-GC (DB-17MS)-MS method is able to screen for atrazine, desethylatrazine, desisopropylatrazine and desethyldesisopropylatrazine at low ppt levels leading to limits of detection of 6.07 ng/L, 2.98 ng/L, 1.25 ng/L and 0.47 ng/L for DDA, DEA, DIA and ATR, respectively, with RSD% smaller than 8 (n=6).

The applicability of the method was then evaluated for a complex mixture of EDCs and pharmaceuticals with a wide variety in polarity. It could be concluded, that this technique is only beneficial for very polar analytes (log $K_{o/w} < 2$). When less polar analytes are determined, they are preferably present in PDMS leading to lower recoveries in the EA extract.

2 Synthesis of new monolithic phases as extraction medium

2.1 Introduction

The limited applicability of SBSE for the analysis of polar analytes is caused by the apolar nature of PDMS as is described in more detail in Chapter II.4.3. One way to improve the extraction for polar solutes is to develop other sorbents.

Recently, more and more research has been done in this respect presenting new polymeric materials coated on stir bars. Some examples are, as described in Chapter II.4.3, the development of stir bars coated with PDMS/β-cyclodextrin [29], restricted access material (RAM) [30], polyurethane foams [31], or monolithic materials [32-34].

The use of monolithic materials as coating on the stir bar is an interesting approach, since the properties of the material vary according to the used monomer and cross-linker. As a consequence, the extraction capabilities of this type of material can be optimized and fine-tuned for the extraction of polar analytes, by changing the monomer and cross linker.

Monolithic material was first introduced by Hjerten et al. in 1989 and since then it gained in popularity [35]. Since monoliths contain a network of interconnected pores with sizes in the low micrometer range, monolithic materials are highly porous and they possess good permeability. The synthesis of such materials requires only one-step polymerization reaction and a simple post-treatment procedure. Furthermore, the porous structure and surface properties are usually tuneable [36].

Monolithic materials are widely employed as stationary phase in HPLC [37], capillary HPLC [38] and capillary electrochromatography [39]. The less common functions of monolithic materials include supports for solid phase and combinatorial synthesis [40], scavenger [41], carriers for immobilization of enzymes [42], in-tube SPME material [43] as well as solid phase extraction material [44].

Recently, monoliths as coating for stir bars were presented by Huang et al.. The monomer and/or crosslinker were varied, leading to three types of monoliths, each with different extraction capabilities. The first type was prepared with octyl methacrylate as monomer and ethylene dimethacrylate as crosslinker. This type was succesfully used for the determination of the apolar PAHs in seawater samples [32]. However, their extraction efficiency for polar analytes was limited. Therefore, a second monolithic material poly(methacrylic acid stearyl ester-ethylene dimethacrylate) was synthesized and used for the analysis of steroid sex hormones in urine samples [33]. As a variation on the latter monolithic material, 4-vinylpyridine was used as monomer with the same crosslinker. This type of monolithic material was used for the determination of phenols in lake water [34]. The analytes extracted by the monolithic stir bars were always recovered by liquid desorption followed by LC-UV analysis.

A novel type of monolithic material was synthesized. A combination of 4-vinylpyridine (VP) and acrylamide (AA) was used as monomers and N,N'methylene bisacrylamide (Bis) as crosslinker. This new material poly(AA-VP-Bis) was initially used for analysis EDCs in aqueous samples but the performance, in terms of robustness, were unacceptable. Therefore, evaluation was done by the analysis of the headspace of coffee. After extraction, thermal desorption was carried out, directly followed by GC-MS analysis. The extraction efficiency of the monolithic stir bar was compared to the conventional PDMS stir bars.

2.2 Experimental

2.2.1 Chemicals

Acrylamide (AA) and 1-dodecanol were obtained from Fluka (Bornem, Belgium). 4-vinylpyridine (VP) and AIBN were purchased from Aldrich (Bornem, Belgium). N,N'-methylene bisacrylamide (Bis) was from Sigma (Bornem, Belgium). DMSO and methanol (HPLC grade) were purchased from Sigma-Aldrich (Bornem, Belgium).

2.2.2 Preparation of the monolithic material

The poly(acrylamide – vinylpyridine – N,N'methylenebisacrylamide) monolithic material (poly(AA-VP-Bis)) was synthesized by a heat-initiated polymerization method described by Fan et al. [45]. First, the polymerization mixture consisting of monomer acrylamide (AA) (7.9 wt%), 4-vinylpyridine (VP) (8.1 wt%,), crosslinker N,N'-methylene bis(acrylamide) (Bis) (8.9 wt%), porogenic solvent DMSO (52.3 wt%) and dodecanol (22.8 wt%), initiator AIBN (1 wt% of monomer and crosslinker) was placed in an ultrasonic bath until a clear solution was obtained. Then, the solution was purged for 3 min with nitrogen to remove oxygen. Subsequently, vials with a volume of 150 µL (Alltech, Lokeren, Belgium) were filled with this solution, sealed and the reaction was initiated at 60 °C for 18 h. The monoliths were taken out of the inserts and overnight Soxhlet extraction with methanol was performed to remove the unreacted component and porogenic solvent. The total mass of each monolith was approximately 20 mg. This corresponds to the mass of a PDMS stir bar of 25 µL.

2.2.3 Sample preparation

The headspace of coffee was invesigated with the new monolithic material and with commercial stir bars for sorptive extraction (Twister^{TM}). The latter were purchased from Gerstel (Gerstel GmbH, Mülheim an der Ruhr, Germany). They consist of a 10 mm length glass-encapsulated magnetic stir bar, coated with 25 µL of PDMS (0.5 mm coating). Stir bars were conditioned for 2 h at 300°C under a constant helium flow and kept in 2 mL vials before use as indicated by the manufacturer. The monolithic material was conditioned for 2 h at 250°C under a constant helium flow and kept in 2 mL vials before use.

The static headspace extraction was carried out using 10 mL of coffee (300 mg coffee powder in 10 mL water) (Douwe Egberts, Dessert) at 60°C for 1 h. Afterwards, the monolith and PDMS stir bars were analysed by TDS-GC-MS.

2.2.4 Instrumentation

The poly(AA-VP-bis) monolithic material was characterized using scanning electron microscopy (SEM), fourier transform infrared spectroscopy (FT-IR) and thermogravimetric analysis.

Scanning electron microscopy was performed on a Quantafeg 200 instrument (FEI, Eindhoven, The Netherlands). A Perkin-Elmer 1600 series FT-IR spectrometer was used for fourier transform infrared spectroscopy. The results were in wave numbers (cm^{-1}). Samples were prepared as a thin film (neat) on KBr plate or used directly with the Horizontal Attenuated Total Reflection (HATR) adaptor. The measurement of the thermal stability was carried out on a thermogravimetric analyser (TGA/SDTA851, Mettler Toledo).

GC-MS analyses were performed on an Agilent 6890 gas chromatograph – 5975 mass spectroscopic detection combination (Agilent Technologies, Little Falls, DE, USA) equipped with a programmed temperature vaporization inlet (CIS-3, Gerstel GmbH, Mullheim, Germany). Thermal desorption was carried out using a TDU unit (Gerstel GmbH, Mullheim, Germany) mounted on the GC via the CIS-3 inlet. The stir bar was placed in a glass tube of 60 mm L, 6 mm OD and 5 mm ID. Splitless thermal desorption was performed by programming the TDU from 30°C (0.2 min) to 250°C (7 min) at a rate of 30°C/min with a helium flow rate of 50 mL/min. The analytes were cryo-focussed in the CIS-3 inlet filled with quartz wool at -125°C using liquid nitrogen. Splitless injection was performed by ramping the CIS-3 from -125°C (0.10 min) to 280°C (5 min) at a rate of 12°C/s.

The analyses were performed on a DB-VRX fused silica capillary column of 20 m L, 0.18 mm ID and a phase thickness of 1.00 μm (Agilent Technologies, Folsom CA, USA). The oven was programmed from 40°C (5 min) to 250°C (4 min) at 8°C/min. Analysis was carried out in constant flow mode at 1 mL/min. Detection was carried out in the scan mode. The transfer line, ion source and quadrupole analyser temperatures were set at 280°C, 230°C and 150°C respectively. Electron ionisation mass spectra were recorded at 70eV electron energy with an ionisation current of 34.6

μA. Data acquisition, instrument control and data analysis were performed by ChemStation software (G1701CA, version C.00.00, Agilent Technologies).

2.3 Results and discussion

2.3.1 Preparation of poly(AA-VP-Bis)

The poly(acrylamide – vinylpyridine – N,N'methylenebisacrylamide) monolithic material was synthesized by an in-situ radical polymerization reaction described by Fan et al. [45]. The structures of the monomers, cross-linker and the resulting monolithic material poly(AA-VP-Bis) are shown in **Figure IV.14**.

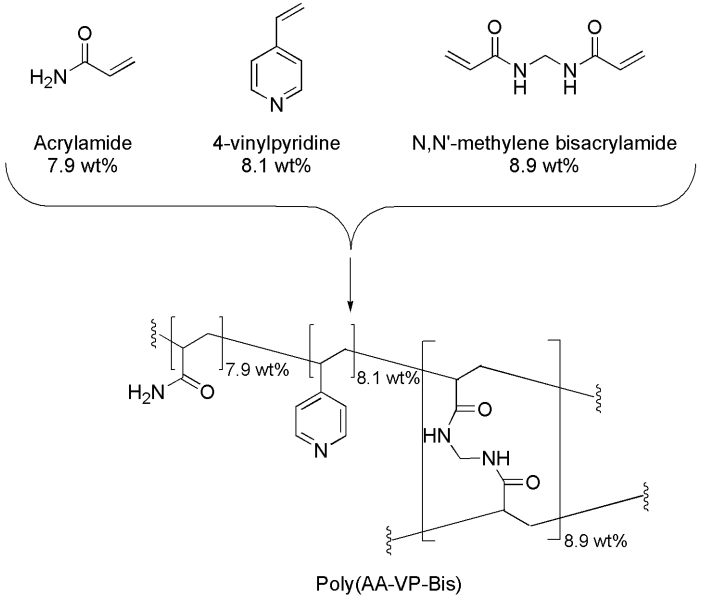


Figure IV.14: Structures of the monomers, crosslinker and the resulting monolithic material poly(AA-VP-Bis).

These monomers were chosen due to their polarity, so that the resulting monolith would be able to extract more polar analytes.

The synthesized monolith was characterized by FT-IR. The resulting spectrum is shown in **Figure IV.15**.

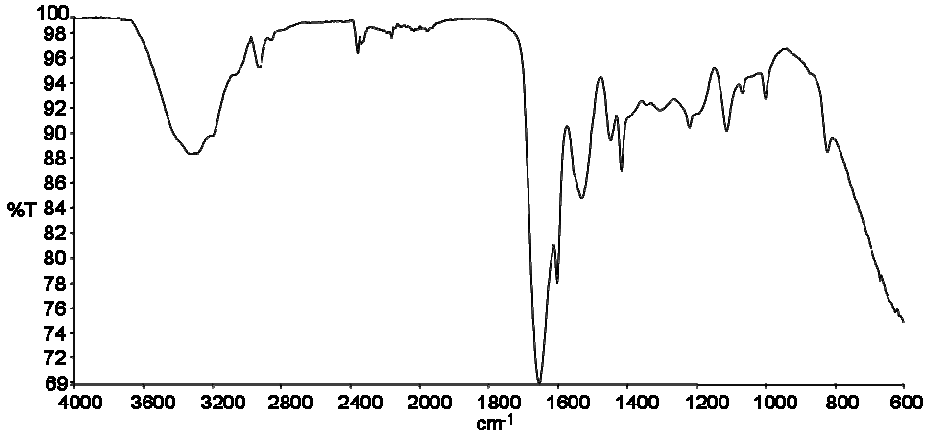


Figure IV.15: FT-IR spectrum of the monolith poly(AA-VP-Bis).

The bands at 3200-3600 cm^{-1} and 1668 cm^{-1} are characteristic of the N-H and C=O stretching frequency of acrylamide, respectively, and the band at 1590 cm^{-1} is indicative for the presence of pyridyl groups.

The morphology of the monolith was studied using scanning electron microscopy (SEM). The resulting pictures are presented in **Figure IV.16**.

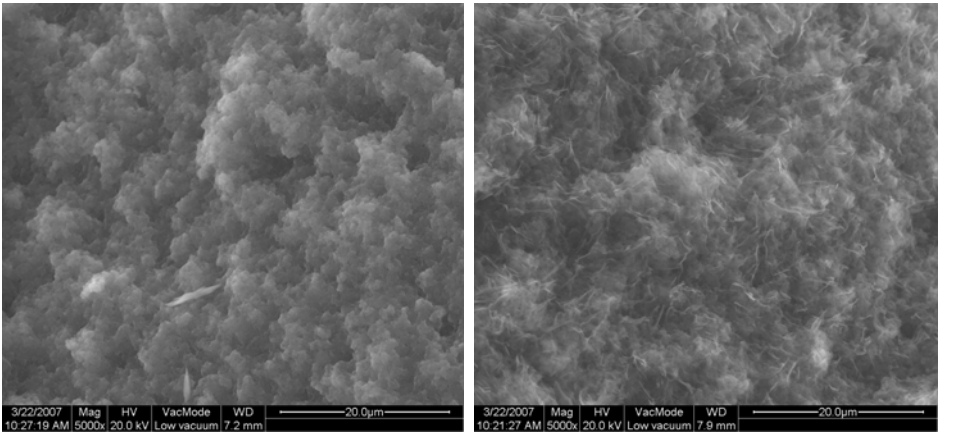


Figure IV.16: SEM images of poly(AA-VP-Bis) monolithic material.

In these pictures, the interconnected skeletons and interconnected textural pores of the monolith can be easily observed.

The thermal stability of the monolith was evaluated by TGA analysis. The results are shown in **Figure IV.17**.

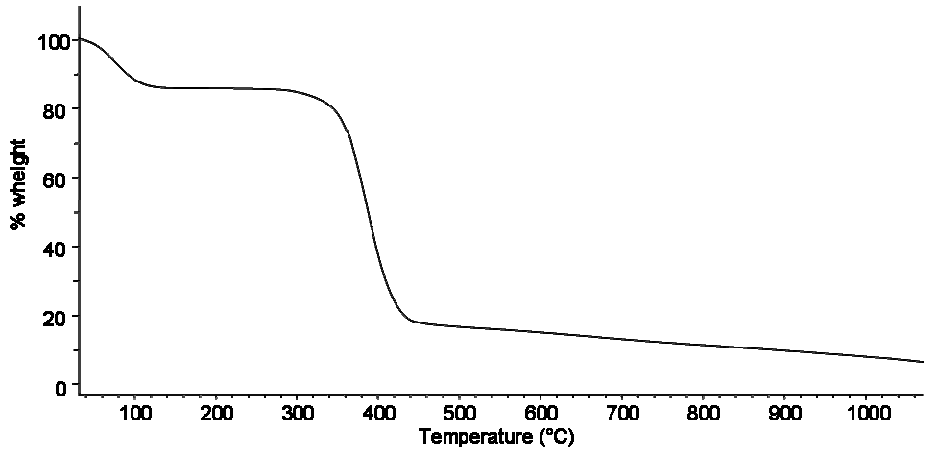


Figure IV.17: TGA analysis of poly(AA-VP-Bis) under inert N_2 atmosphere.

It can be seen that the monolith is stable until 300°C. At higher temperatures, thermal degradation occurs.

2.3.2 Evaluation of poly(AA-VP-Bis)

The extraction capabilities of the monolithic material are evaluated and compared to the extraction efficiency of the conventional stir bars (25 µL) for the headspace analysis of coffee (Douwe Egberts, Dessert). Both materials are shown in **Figure IV.18**

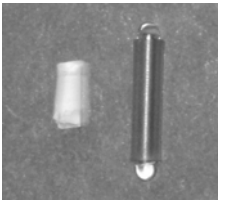


Figure IV.18: Pïcture of the monolithic material (left picture) and the PDMS stir bar (right picture)

This monolithic material was first used for the enrichment of EDCs in aqueous samples, but the performance, in terms of robustness, were unacceptable due to limited stability of the material. Consequently, this monolithic material was further

evaluated in headspace; more specifically it was used for the static headspace extraction of coffee.

Static headspace extraction of coffee was carried out using 10 mL of coffee (300 mg coffee powder in 10 mL water) at 60° for 1 h. Afterwards, the monolith and the PDMS stir bar were analysed by TDS-GC-MS. The results are shown in **Figure IV.19**.

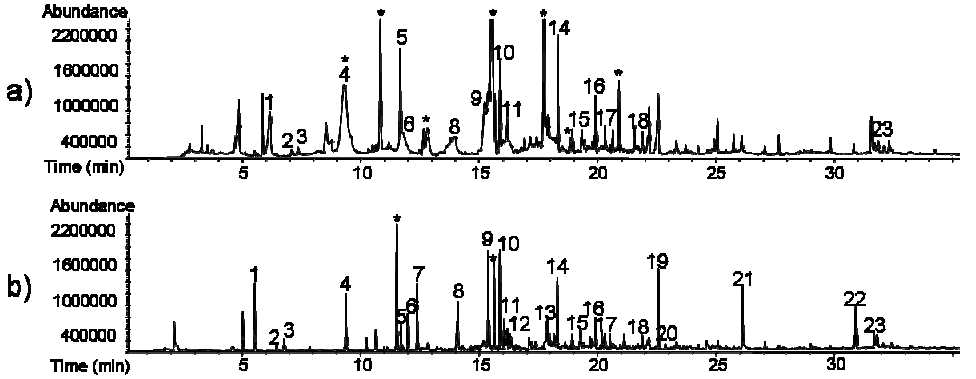


Figure IV.19: Total ion chromatograms of the headspace analysis of coffee with the monolithic material (a) and PDMS (b). The compounds are denoted in **Table IV.6**.

Table IV.6: Peak allocation of the static headspace analysis of coffee shown in **Figure IV.19**.

	Peak allocation
Tetrahydrofuran	1
3-methylbutanal	2
2-methylbutanal	3
Pyridine	4
Methylpyrazine	5
Furfural	6
2-furanmethanol	7
2.6-dimethylpyrazine	8
5-methyl-2-furfural	9
2-furanmethanol acetate	10
2-ethyl-6-methylpyrazine	11
2-ethyl-5-methylpyrazine	12

	Peak allocation
3-ethyl-2.5-dimetylpyrazine	13
Nonanal	14
Benzoic acid	15
1-furfurylpyrrole	16
Decanal	17
4-ethyl-2-methoxyphenol	18
2-methoxy-4-vinylphenol	19
Decanoic acid	20
Dodecanoic acid	21
Caffeine	22
n-Hexadecanoic acid	23
Degradation products of the stir bar material	*

From these results, it can be seen that the extraction capabilities of PDMS are much better. Some compounds such as caffeine are not extracted by the monolithic material. In addition, the monolithic material generates a lot of different bleeding products, all based on pyridine (indicated with * in **Figure IV.19**), which complicates identification and quantification and necessitates the use of mass spectrometry. PDMS, on the other hand, leads to small amounts of degradation products that are well known and can easily be identified. In conclusion, the use of monoliths as extraction medium in combination with thermal desorption suffers from some major drawbacks.

2.4 Conclusion

A new type of polymer is introduced as extraction medium in order to improve the extraction of polar analytes from aqueous samples. The monolithic material was prepared using an in-situ polymerization of acrylamide, 4-vinylpyridine and N,N'-methylene bisacrylamide. After the synthesis, the material was characterized using SEM analysis, FT-IR spectroscopy and TGA analysis.

In order to evaluate the extraction capabilities of the monolithic material, it was first used for the analysis of EDCs in aqueous samples, but due to limited stability of

the material, the results were very disappointing. Therefore, the material was further evaluated in headspace such as static headspace analysis of coffee. The results were then compared to those obtained with conventional PDMS. The extraction efficiency of the monolith was less compared to PDMS. This is ascribed to the adsorption mechanism rather than to the sorptive mechanism occurring on PDMS. Furthermore, the degradation products of the monolith disturb the background, thereby complicating identification and quantification.

In conclusion, PDMS delivers better results and is therefore preferably used as extraction medium.

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CHAPTER V
DEVELOPMENT OF A MORE SELECTIVE SAMPLE PREPARATION TECHNIQUE FOR EDCs*

A library of possible mimics of the estrogen receptor was prepared. The affinity of the members towards 17-β-estradiol was evaluated using two different screening techniques. The first one is based on affinity liquid chromatography. An affinity column was prepared where estradiol or testosterone was bound to the stationary phase. The synthesized receptors show affinity, but no selectivity towards 17-β-estradiol. After the competition experiment, where 17-β-estradiol was present in the mobile phase, it was concluded that the separation mechanism is based mostly on partition and not on affinity. Consequently, the estradiol and testosterone column are not suitable as screening technique for the synthesized library.

The second screening technique is based on solid phase extraction. The extraction efficiency of two library members was compared to that of a commercially available SPE material Oasis HLB® for laboratory water and hospital effluent, both spiked with EDCs.

* 'Towards a new SPE material for EDCs: Fully automated synthesis of a library of tripodal receptors followed by a fast screening via affinity LC'

S. Van der Plas, E. Van Hoeck, F. Lynen, P. Sandra, A. Madder, submitted to Eur. J. Org. Chem.

1 Introduction

Most of the EDCs disturb the endocrine system by interacting with the hormone-binding domain (HBD) of the human estrogen receptor (hER) which exists in two isomers, hERα and hERβ. The concentrations of these two types of receptor are different throughout the body as well as their biological role. Since the composition of both HBDs is very similar, most EDCs interact in the same way with both isomers. The current work is based on the α-isomer of hER and it will further be referred to as hER.

In theory, a synthetic version of this hormone-binding domain of the human estrogen receptor could show the same affinity for EDCs as the naturally occurring one. Such a synthetic receptor can only be successful if two conditions are fulfilled. Firstly, the 3D-structure should be similar to that of the hormone-binding domain of the naturally occurring human estrogen receptor. Secondly, the amino acids that are responsible for the interaction between the EDCs and the hormone-binding domain of the human estrogen receptor should be present.

In the last decade, the interactions between the hormone binding domain of estrogen receptor and different EDCs were investigated based on X-ray structures [1]. A schematic representation of the interactions of HBD of the αhER with 17-β-estradiol is depicted in **149Figure V.1**.

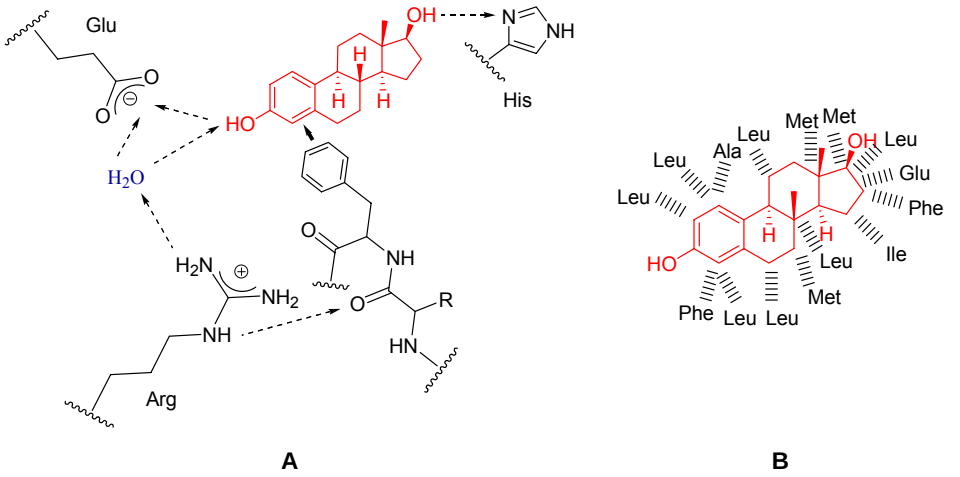


Figure V.1: Schematic representation of hydrogen bonding network (A) and the hydrophobic interactions (B) between the amino acids of the HBD of the hER and its natural ligand estradiol.

From this figure, it can be seen that two types of interactions, namely hydrogen bonding and Van der Waals interactions are necessary for binding estradiol in the HBD of the hER. The hydrogen bonding network consists of the hydroxyl functions of 17-β-estradiol, glutamic acid (Glu), arginine (Arg), histidine (His), the backbone of phenylalanine (Phe) and a water molecule. This network is supported by a hydrophobic network, which is formed by the aromatic part of 17-β-estradiol and the side chain of phenylalanine. Furthermore, 17-β-estradiol interacts with various apolar side chains of leucine, isoleucine, phenylalanine, alanine and methionine. The structures of these amino acids are given in **Figure V.2**.

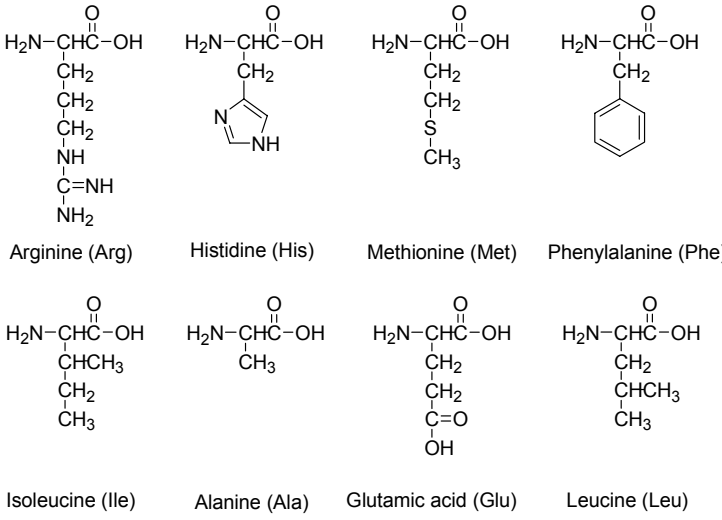


Figure V.2: Structures of the amino acids which are responsible for the interaction between the HBD of the hER and 17-β-estradiol.

After the synthesis of a mimic of the HBD of the hER, its affinity towards 17-β-estradiol and other EDCs needs to be evaluated.

In general, screening for affinity has been done in many different ways. A first possibility is incubating the synthesized molecule with a fluorescently labeled or radioactive ligand. In addition, chromatographic techniques such as affinity chromatography or affinity electrophoresis can also be used as screening techniques.

Liskamp et al. labeled the dipeptide D-alanine-D-alanine, which is a precursor for the bacteria cell wall, with a fluorescent group in the search for new possible antibiotics [2]. Consequently, receptors that are able to bind the dipeptide, can play an important role in the next generation of antibiotics. The labeled dipeptide is shown in **Figure V.3**.

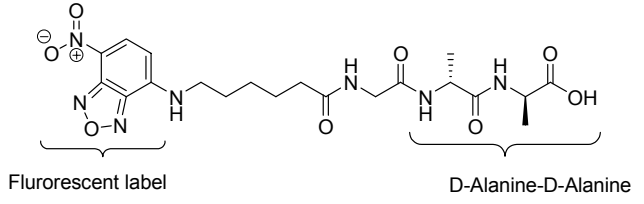


Figure V.3: Fluorescent labeled D-alanine-D-alanine

The labeled ligand was incubated with beads on which possible receptors are immobilized. Afterwards, beads were washed and evaluated with the use of a fluorescence microscope. The most fluorescent beads were isolated and further evaluated for possible antibiotic activity.

The use of a ligand with a fluorescent label presents some drawbacks. Firstly, the fluorescently labeled molecule is often not readily available, which for most applications entails that a fluorescent ligand must be synthesized. This is not always successful and furthermore, the ligand becomes very different from the unlabeled substrate. As a consequence, the fluorescent label can also be responsible for the measured interaction. By evaluating the interactions caused by the fluorescent label itself, this drawback can be overcome. Usually, these interactions are investigated either by incubating the fluorescent label with the possible receptor or by performing a second screening with a different label [2].

Another screening technique utilizes radioactive [^{3}H]-labeled compounds. With this approach, the changes to the ligand are minor but the molecule is still detectable at very low concentrations. Tozzi et al. used [^{3}H]-labeled 17-β-estradiol for the evaluation of the binding properties of synthesized peptides towards 17-β-estradiol, with the aim of making a synthetic affinity column [3]. The peptides were synthesized using solid phase peptide synthesis. The evaluation was performed by incubating the beads with tritium labeled estradiol. Afterwards, the beads were spun down, the supernatant was added to the liquid scintillation cocktail and the radioactivity was measured. The higher the measured radioactivity, the lower the affinity is towards 17-β-estradiol.

Affinity chromatography is a very powerful screening technique. It is based on a highly specific interaction such as that between antigen and antibody or receptor and ligand. Normally, the antibody or the receptor is immobilized on a stationary phase, and the antigen or ligand is present in the mobile phase. The stronger the interaction, the longer the retention time of the antigen or the ligand will be. Lynen et al. used an affinity column based on vancomycin to screen a combinatorial library of polypeptides [4]. The longer the retention times of the library members, the higher is

the affinity towards vancomyin. An important disadvantage of this technique is that the packing material of the column will also interact with the library members and will thus influence the retention times. Furthermore, these affinity columns are rather expensive. The last few decades, scientific research has therefore tried to substitute them with synthetic systems with similar recognition properties [3].

Affinity capillary electrophoresis is a solution based screening technique. The major advantage is that the observed interaction is now caused only by the receptor and there are no contributions of the solid phase on which it was attached. In affinity capillary electrophoresis, the change in electrophoretic mobility between a free ligand and a complex of the ligand with a receptor dissolved in the background is measured. This technique was utilized by Lynen et al. for the screening of a library of oligopeptides for their affinity to vancomycin [5].

In this contribution, a library of possible mimics of the estrogen receptor was prepared. The affinity of the members towards 17-β-estradiol was evaluated using two different orthogonal screening techniques. The first one is based on affinity liquid chromatography. An affinity column was prepared where estradiol or testosterone was bound to the stationary phase. Both columns were evaluated as affinity column for the synthesized library. The second screening technique is based on solid phase extraction. Both techniques were first evaluated using a peptide of which the affinity towards 17-β-estradiol has already been described [3]. Finally, the extraction efficiency of one library member was compared to the extraction efficiency of commercially available SPE material Oasis HLB® for the analysis of spiked laboratory water and spiked hospital effluent.

2 Experimental

2.1 Chemicals

Neat certified endocrine disrupting standards were purchased from different sources. 17-β-estradiol-d_3 and bisphenol A were supplied by Aldrich (Bornem, Belgium). Estrone and 17-β-estradiol were purchased from Sigma (Bornem, Belgium). Testosterone, diethylstilbestrol, 17-α-ethinyl estradiol and tetrahydrofuran (THF) were supplied by Fluka (Bornem, Belgium). The chemical structures of the endocrine disrupting chemicals are given in **Figure V.4**.

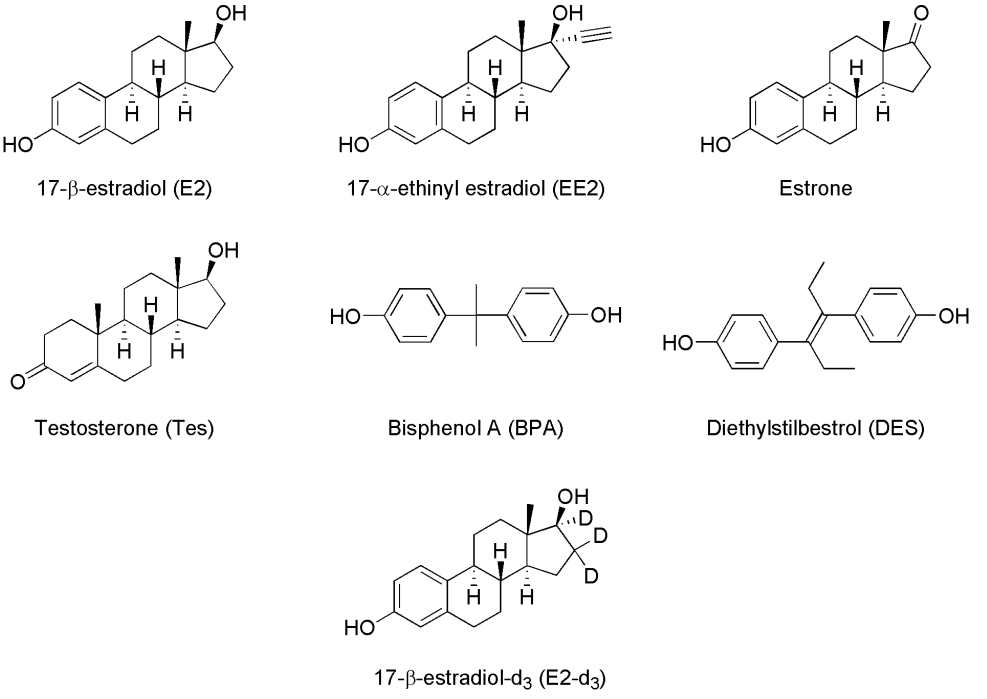


Figure V.4: Structures of the EDCs

Tentagel, Merrifield, Clear and PEGA were purchased from NovaBiochem (Merck KGaA, Darmstadt, Germany). Aminopropyl silicagel (Nucleosil, 5µm, 100 Å) was supplied by Macherey-Nagel (Düren, Germany). Oasis HLB® SPE material

was supplied by Waters (Milford, MA, USA). Water, methanol (MeOH) and acetonitrile (ACN) (all MS grade) were supplied by Biosolve (Valkenswaard, The Netherlands). Amonium acetate (NH_4OAc) and ammoniak (NH_3) were purchased from Sigma. Stock solutions of each individual compound were prepared in acetonitrile at a concentration of 1 mg/mL. This solution was stored at 4°C and used to prepare the spiking solutions.

2.2 Phase preparation

2.2.1 Affinity LC

Synthesis of the new stationary phases based on estradiol and testosterone

The synthesis of phases was carried out by Drs. S. Van der Plas of the group of Prof. Dr. A. Madder at the Laboratorium of Organic and Biomimetic Chemistry of our department.

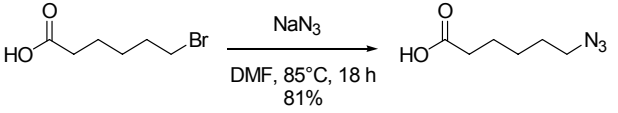
The different steps in the synthesis are described. Each reaction was verified using the following techniques:

NMR spectra were recorded at 500 MHz for proton and at 75 MHz for carbon nuclei in chloroform-*d* ($CDCl_3$). Chemical shifts are reported in units of parts per million (ppm), referenced relative to the residual ^{1}H or ^{13}C peaks of chloroform-*d*: ^{1}H 7.26 and ^{13}C 77.16. The following abbreviations were used to explain the multiplicities: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet; br, broadened.

Infrared spectra (FT-IR) were recorded on a Perkin-Elmer 1600 series FT-IR spectrometer and are reported in wave numbers (cm^{-1}). Samples were prepared as a thin film (neat) on KBr plate or used directly with the Horizontal Attenuated Total Reflection (HATR) adaptor. The following abbreviations were used to explain the width in the FT-IR spectra: s, small; m, medium; br, broad.

Mass Spectra were recorded on LCQ $MS^{(n)}$ (Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionization source.

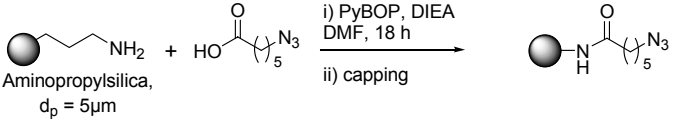
Synthesis of azidohexanoic acid



Bromohexanoïc acid (6 g, 31 mmol, 1 eq) was dissolved in 20 mL N,N-dimethylformamide (DMF). After adding NaN_3 (4 g, 62 mmol, 2 eq) the solution was refluxed for 18 h in an oil bath temperature of 85°C. After cooling down the solution, DMF was evaporated under vacuum to give an oil that was redissolved in DCM. This organic phase was then extracted 3 times with a 0.1 M HCl solution. After drying the organic phase on $MgSO_4$ and evaporation, 3.96 g of a light yellow oil was obtained (81% yield).

Molecular Formula: $C_6H_{11}N_3O_2$
MW: 157.09 g/mol
TLC: with DCM and a drop of acetic acid: ratio to front: 0.17
FT-IR: (KBr-plate): 3075 (br.), 2939 (m), 2866 (m), 2093 (s), 1706 (s), 1254 (m), 910 (m), 731 (s) cm^{-1}.
MS: (m/z): 156.2 g/mol
^{13}C-NMR (APT): (75 MHz, $CDCl_3$): δ 179.5 (C), 51.2 (CH_2), 33.8 (CH_2), 28.5 (CH_2), 26.1 (CH_2), 24.1 (CH_2) ppm.
^{1}H-NMR (COSY): (500 MHz, $CDCl_3$): δ 10.37 (1H, br. s), 3.26 (2H, t, *J* = 6.9 Hz), 2.86 (2H, t, *J* = 7.4 Hz), 1.69-1.64 (2H, m), 1.62-1.58 (2H, m), 1.45-1.39 (2H, m) ppm.

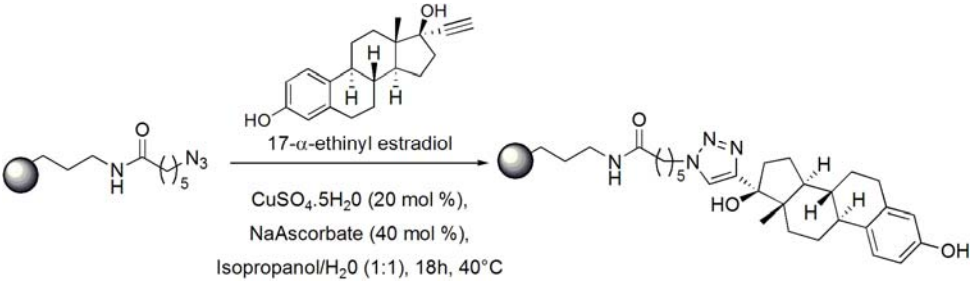
Coupling of azidohexanoic acid to aminopropyl silicagel



The silicagel material (2 g, max. loading 0.73 $mmolg^{-1}$) was suspended in DMF (10 mL) and azidohexanoic acid (691 mg, 4.4 mmol, 3 eq) was added to this suspension. After addition of PyBOP (2.3 g, 4.4 mmol, 3 eq) and DIEA (1.53 mL, 8.8 mmol, 6 eq) the suspension was shaken for 18 h. Subsequently, the solution was drained and the remaining powder was washed thoroughly. After capping, the ninhydrine test gave a colourless result, indicating that all amine functions have reacted.

FT-IR. (KBr-plate): 3414 (br.), 2104 (s), 1642 (m), 1111 (br), 810 (s)

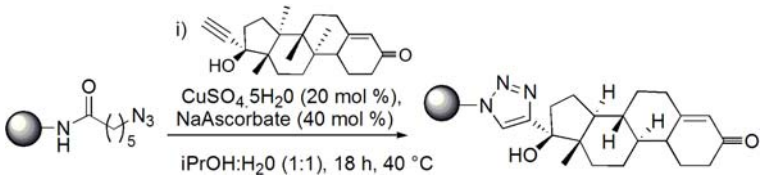
'Clicking' of 17-α-ethinylestradiol to the modified aminopropyl silicagel



The silicagel material (6 g) was suspended in an isopropanol/H_2O (60 mL in a ratio of 1:1) solution. Ethinylestradiol (4.45 g, 15 mmol, 1 eq) was added to this suspension followed by $CuSO_4.5H_2O$ (749 mg, 3 mmol, 20 mol %) and Na-ascorbate (1.2 g, 6 mmol, 40 mol %). The resulting orange solution was stirred gently in an oil bath at 40 °C. After overnight reaction, the suspension was filtered and the remaining silicagel material was washed three times with MeOH, three times with ethylenediaminetetra-acetic acid ($EDTA_{aq}$) and three times with ether. All the azide functions had reacted as witnessed by the disappearance of the azide absorption band (~ 2100 cm^{-1}) in the FT-IR-spectrum. Finally, the phase was dried at 60 °C for 12 h.

FT-IR: (KBr-plate): 3399 (br. s), 2936 (s), 1670 (s), 1050 (br. s), 800 (s)

'Clicking' of 19-norethindrone to the modified aminopropyl silicagel



The silicagel material (1 g) was suspended in an isopropanol/H_2O (10 mL in a ratio of 1:1) solution. 19-norethindrone (756 mg, 2.5 mmol, 1 eq) was added to this

suspension followed by $CuSO_4.5H_2O$ (125 mg, 0.5 mmol, 20 mol %) and Na-ascorbate (198 mg, 1.0 mmol, 40 mol %). The resulting orange solution was stirred gently in an oil bath of 40 °C. After overnight reaction, the green suspension was filtered and the remaining silica material was washed three times with MeOH, three times with $EDTA_{aq}$ and three times with ether. Finally, the modified silicagel material was dried at an oven temperature of 60 °C for 12 h.

The filtrate was extracted three times with DCM. The organic phases were pooled, extracted once with a 10 % Na_2CO_3 solution and subsequently dried over Na_2SO_4. After filtration, the organic phase was evaporated under reduced pressure to yield 524 mg of a white powder. LC-MS and ^{1}H-NMR analysis showed that this was pure norethindrone. From this, it was calculated that 232 mg (0.744 mmol) was coupled to the silica; resulting in a final loading of 0.609 mmol/g.

All the azide functions had reacted as witnessed by the disappearance of the azide absorption (~ 2100 cm^{-1}) in the FT-IR-spectrum.

FT-IR: (KBr-plate): 3430 (br. s), 2947 (m), 1647 (s), 1092 (br. s), 802 (s) cm^{-1}

Packing of columns with the new stationary phases (150 mm L x 2.1 mm ID)

Column packing was performed using the slurry packing method with a Haskel air driven pump (Burbobank, CA, USA) [6]. The set-up is shown in **Figure V.5**.

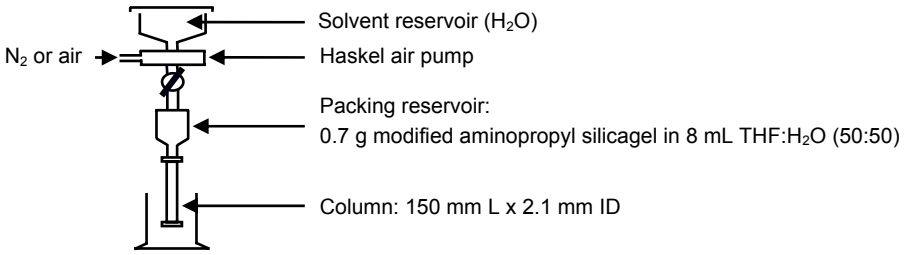


Figure V.5: Set-up for packing of columns by the slurry method

The slurry solvent was THF:water (50:50) and the packing solvent was deionized water. Both were degassed. About 0.7 g of the derivatized silica was slurried in 8 mL

of slurry solvent in an ultrasonic bath followed by packing at 450 bar. The columns were 150 mm in length and 2.1 mm ID.

Packing of the capillary column (50 mm L x 0.25 mm ID)

A capillary column (50 mm L x 0.25 mm ID) with external frits was packed using the slurry packing method. The set-up is presented in **Figure V.6**.

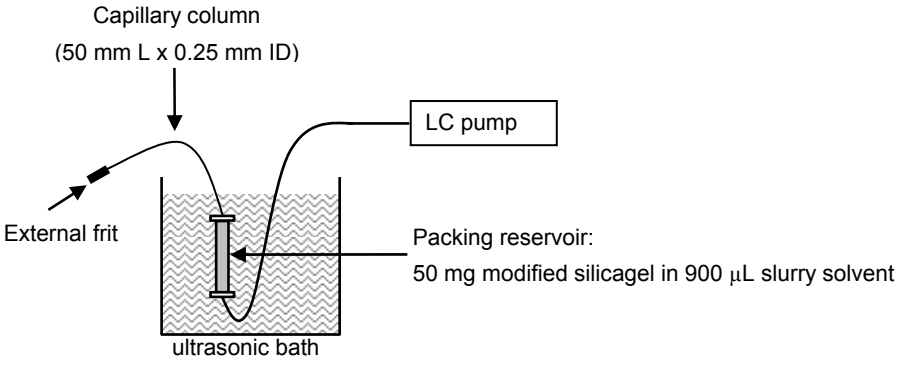


Figure V.6: Set-up for the packing of capillary columns using the slurry method.

The slurry solvent was THF:water (50:50) and the packing solvent was deionized water, both were degassed. About 50 mg of the derivatized silicagel was slurried in 900 µL of solvent in an ultrasonic bath followed by packing at 300 bar.

2.2.2 SPE procedure

An empty 96 SPE well plate was obtained from Chrompack (Varian, Brussels, Belgium). 10 mg of each resin that needs to be evaluated was placed in one of the SPE cartridges of the well plate. The resin was held in place with another filter and a rind. A schematic overview is given in **Figure V.7**.

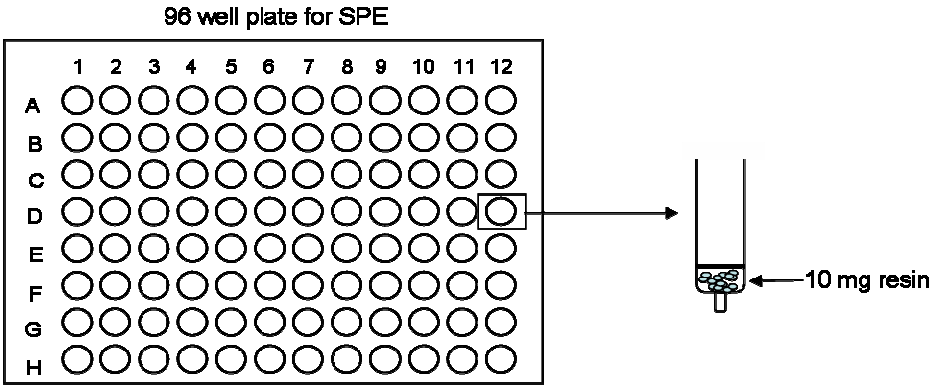


Figure V.7: Schematic overview of the SPE well plate

The affinity of the resins was evaluated using the following procedure. First, the resins were pre-swelled with 900 µL 10 mM NH_4OAc water, adjusted with NH_3 to obtain pH 7.4. Afterwards, the cartridge was loaded with 900 µL of the same water spiked with EDCs, resulting in a concentration of 10 mg/L (ppm) for each EDC in the water. The flow-rate was approximately 0.5 mL/min. Finally, 100 µL of a 100 mg/L (ppm) solution of 17-β-estradiol-d_3 in water was added to the water effluent after the loading, followed by analysis with LC-MS. The concentrations of the EDCs in the water effluent are determined using the internal standard 17-β-estradiol-d_3. After the SPE extraction, the cartridges were rinsed with MeOH to remove the extracted EDCs from the resin. The resins could be re-used after this procedure.

2.3 Instrumentation

LC-MS analyses were carried out on an Alliance 2690 LC system equipped with an on-line degasser and an autosampler (Waters, Milford, MA, USA).

The affinity LC analyses were carried out on the home-made estradiol or testosterone column (150 mm L x 2.1 mm ID, 5 µm d_p). The column was thermostated at 37°C. The mobile phase consisted of 10 mM NH_4OAc in water and methanol. The pH of water was adjusted to 7.4 with NH_3. Different gradients were evaluated and the best results were obtained using the following gradient. First, the mobile phase consisted of 100% 10 mM NH_4OAc for 10 min. Then, a linear gradient

to 100% MeOH was programmed in 10 min. Finally, this composition was maintained for 15 min. The flow rate was 0.2 mL/min and the injection volume was 10 µL, which corresponds with approximately 250 mg/L (ppm).

The competition experiment was carried out with the capillary column (50 mm L x 0.25 mm ID, 5µm d_p) packed with the estradiol stationary phase. Approximately 0.5 µL of 125 mM of the synthesized receptor and 150 mM tetraglycine solution in water was analyzed isocratically at 70% MeOH in 10 mM NH_4OAc/NH_3 in water (pH 7.4) The analysis was carried out at room temperature. The flow rate was 2 µL/min delivered with a syringe pump.

The effluent obtained after the SPE procedure was separated on a Luna-C18 column (150 mm L x 2.1 mm ID, 5 µm d_p) (Phenomenex, Torrance, CA, USA). The column was thermostated at 20°C. The mobile phase consisted of acetonitrile and water. A linear gradient program was used from 40% acetonitrile to 60% acetonitrile in 15 min. Afterwards the mobile phase was switched directly to 100% acetonitrile. This mobile phase composition was then maintained for 5 min. The flow rate was 0.2 mL/min and the injection volume was 10 µL.

Ultraviolet detection was carried out using a Waters 2487 dual λ absorbance detector (Waters, Milford, MA, USA). Analyses were recorded at 210 and 254 nm for the affinity LC evaluation and at 230 nm for the SPE procedure.

Mass spectrometric detection was carried out using a Quattro Micro system equipped with a Z-spray electrospray source (Micromass Manchester, UK). For the affinity screening and the competition experiment, the mass spectrometer was used in the positive mode. The capillary voltage was set at 3 kV and the cone voltage at 40 V. Mass spectra were recorded in scan mode beween 150 and 550 m/z for the analysis of the Tozzi peptides and tetraglycine and in the scan mode between 850 and 1000 m/z for the analysis of the library members.

For the SPE experiments, the mass spectrometer was used in negative mode. The capillary voltage was set at 2.5 kV and the cone voltage at -40 V. The source temperature and desolvation temperature were for both procedures at 120°C and 350°C, respectively. A cone gas flow of 50 L/h and a desolvation gas flow of 350 L/h were used. Mass spectra for the SPE procedure were recorded in the SIM mode. One

characteristic ion for each compound was selected. The SIM ions are 227.35 for BPA, 267.42 for DES, 269.44 for estrone, 271.45 for E2, 274.45 for E2-d_3, 287.46 for TES and 295.46 for EE2. The dwell time was 100 ms.

Data acquisition, instrument control and data analysis were performed by Masslynx software (version 4.0, Micromass).

3 Results and discussion

3.1 Synthesis of an artificial estrogen receptor

The synthesis of the artificial estrogen receptor was carried out at the Laboratorium of Organic and Biomimetic Chemistry (Prof. Dr. A. Madder) by Drs. S. Van der Plas.

The first criterium for a suitable mimic is the resemblance of the 3D-structure to the original structure of the HBD of hER. In order to create a 3D-arrangement of polypeptide chains, a tripodal scaffold was used. The general structure is given in **Figure V.8**.

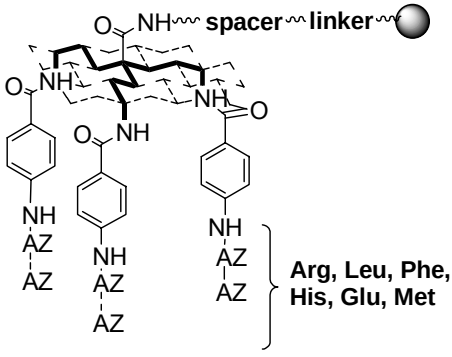


Figure V.8: General structure of the tripodal scaffold.

This tripodal scaffold can adopt a pre-organised 3D-arrangement due to the presence of aromatic interactions between the three arms. This parallel orientation was confirmed by modelling studies using Macromodel V.6.0 (force field MM2). To reduce calculation times, the carboxylic acid was replaced by a methyl group and the three different amine protecting groups were simplified as acetyl groups. **Figure V.9** shows the two energetically most favoured conformations in water.

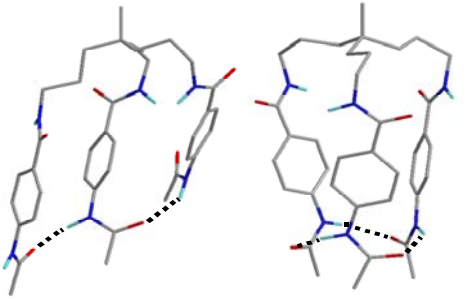


Figure V.9: Two most energetically favoured conformations of the tripodal scaffold in water.

Extra stabilisation of the tripodal scaffold in water is achieved through hydrogen bonding, represented by the dotted lines in **Figure V.9.** From these modelling studies it is clear that some conformational freedom is possible, but a parallel orientation of the three arms can be adopted.

Another feature of the scaffold is the carboxylic acid head group that will function as an anchoring point, allowing attachment to a solid support, for example Tentagel-NH_2.

In order to fulfil the second criterium for a successful artificial receptor the same amino acids as in the X-ray data presented above should be used. From these data, six amino acids were selected to be incorporated into the tripodal backbone. For mimicking the hydrogen bond network, His, Arg and Glu were chosen. The apolar environment of the receptor is mimicked by Leu, Phe and Met.

Since the scaffold is attached to the solid support Tentagel-NH_2, solid-phase peptide synthesis protocols can be employed for the attachment of amino acids to the tripodal scaffold. This has the advantage that for each reaction, a large excess of reagents can be used, driving the reaction to completion and enabling fast reaction kinetics. When the reaction has finished, the excess of reagents can simply be washed away.

A library is generated by attaching two amino acids to each arm of the scaffold. This can lead to a library of 6^6 or 46,656 members. This is almost impossible to synthesise and it is even more difficult to evaluate all these members. In order to limit the amount of library members, a few restrictions were applied. Firstly, all amino

acids are only present once in each library member. Secondly, all the library members where it seems that one strand has traded place with another strand, were left out. This is illustrated in **Figure V.10**.

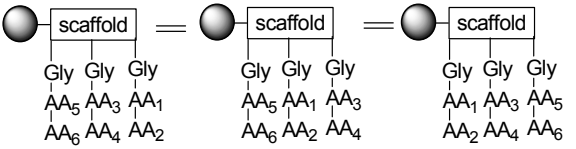


Figure V.10: Limiting the number of synthesized library members.

In this way a library of 120 members was generated. The general structure of these members is shown in **Figure V.8.**

3.2 Evaluation of the synthesized estrogen receptors

3.2.1 Peptides with known affinity towards 17-β-estradiol

Tozzi et al. prepared small polypeptides that could selectively bind 17-β-estradiol and other similar structures in aqueous media [3].

The monomers used for the peptide synthesis were arginine (Arg), serine (Ser), proline (Pro), valine (Val), leucine (Leu), glutamine (Gln), glycine (Gly) and alanine (Ala). These were chosen because they are present in the estradiol binding site of the human steroid binding protein, a transport protein present in the serum of humans. Only alanine does not belong to this sequence, but it substitutes lysine and methionine, which have reactive groups in their side chains. A first library of dipeptides was prepared on Amberlite IRC-50 as solid phase. By incubating the different solid-phase bound members with tritium-labeled 17-β-estradiol, the dipeptide with the best binding characteristics was selected. This dipeptide was then the starting material for a second library, now consisting of tetrapeptides. Again the best tetrapeptide was selected by measuring the radioactivity of the beads. These steps were repeated until the stage of the octapeptide. Tozzi et al. did not provide any proof of the identity and purity of the synthesized pepides. The solid phase used for the synthesis was Amberlite IRC-50, an acrylic acid based polymer that was cross-linked

to a high degree with divinylbenzene. Normally, this resin is used as a cation exchange resin in chromatography and not as solid support in solid phase peptide synthesis.

The affinity of the peptides was evaluated by incubating the peptides with tritium labeled 17-β-estradiol. Afterwards, the beads were spun down, the supernatant was added to the liquid scintillation cocktail and the radioactivity was measured. The higher is the measured radioactivity, the lower the affinity is towards 17-β-estradiol. Non-specific binding of the labeled steroid was evaluated by replacing the beads functionalized with the peptides, with beads completely blocked with ethanolamine. The dissociation constants K_d were determined by adding different amounts of [^{3}H]-17-β-estradiol to the peptides and measuring the radioactivity of the solutions. The values of the dissociation constants of the peptides with the highest affinity towards 17-β-estradiol are given in **Table V.1.**

Table V.1: K_b values towards estradiol and the ratios ($R_{estradiol/testosterone}$) between the 17-β-estradiol bound/free and the testosterone bound/free of the sequences, which showed the best affinity towards 17-β-estradiol.

Peptide	K_d(μM)	$R_{estradiol/testosterone}$
Arg-Ser	32	6
Arg-Ser-Ser-Val	17	10.1
Arg-Ser-Ser-Val-Gly-Ser	12	5.6
Arg-Ser-Ser-Val-Gly-Ser-Gln-Ser	15	3.4

A trend in decreasing dissociation constants can be observed until the hexapeptide, while the octapeptide already has less affinity for estradiol.

The selectivity of the peptides was also evaluated. This was done by repeating the same procedure, but now with tritium labeled testosterone. The selectivity is expressed as the ratio 17-β-estradiol bound/free to testosterone bound/free **(**$R_{estradiol/testosterone}$**)** and they are also listed in **Table V.1.** The higher R, the more selective the peptide is for estradiol in comparison with testosterone. It seems that after the tetrapeptide, the selectivity declines. Because of its good selectivity and affinity properties, the beads containing the tetrapeptide were selected to pack a pre-

concentration column. This was used for the analysis of a mixture of natural and synthetic hormones and good recoveries were obtained.

These results are very promising, since only a linear sequence of amino acids was used, without a complex protein structure. Hence these peptides were used to evaluate the screening techniques developed in this work.

3.2.2 Affinity liquid chromatography

3.2.2.1 Development of new stationary phases using click chemistry

In this contribution, a novel stationary phase will be prepared where 17-α-ethinyl estradiol is bound on the stationary phase. The synthesized library of possible estrogen receptors is disconnected from their solid phase, subsequently they are injected on the affinity column and the retention times are recorded. Furthermore, the selectivity is examined by attaching 19-norethindrone to the stationary phase, thus making a testosterone based column. The same library members are injected and their retention times are compared with those on the estradiol based column.

Synthesis of the new stationary phases

A new approach to synthesize novel stationary phases is by coupling molecules with the desired chemistry to a silicagel support. It is essential that the immobilization method is highly efficient, selective under mild reaction conditions and easy to perform. Click chemistry provides the ideal reactivity profile for this purpose.

The primary reaction of click chemistry is the copper(I) catalyzed [3+2] dipolar cycloaddition between organic azides and terminal alkynes described by Sharpless et al. [7]. The general reaction is demonstrated in **Figure V.11**.

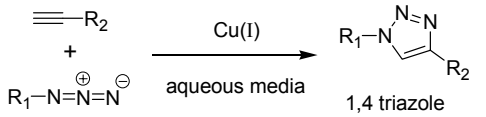


Figure V.11: Primary reaction of click chemistry:
Cu(I) catalyzed addition of an azide to an alkyne.

Applied on the specific situation, it was decided to attach the alkyne-bearing 17-α-ethinyl estradiol (EE2) and 19-norethindrone to an azido-functionalized silicagel material via the Cu(I) catalyzed addition. **Figure V.12** summarizes the synthesis of the stationary phase with EE2 using click chemistry.

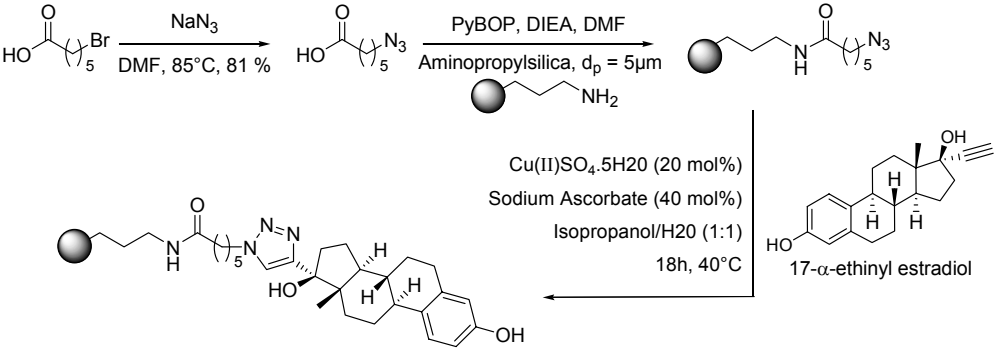
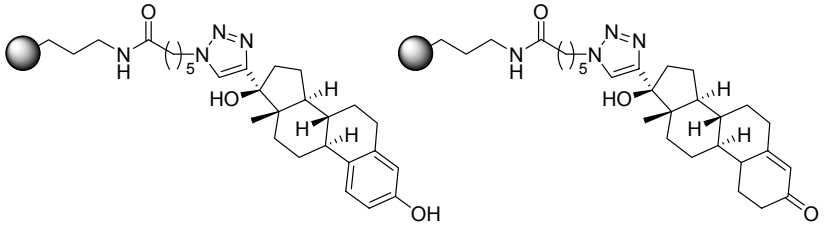


Figure V.12: Synthesis of the stationary phase with 17-α-ethinyl estradiol.

Bromohexanoic acid was converted into its azide analoge by a S_N2 substitution with sodium azide in good yields [8]. Then, PyBOP was added to activate the carboxylic acid and the azide functionalized aminopropyl silicagel was prepared. The subsequent click reaction was performed in a 1:1 isopropanol/H_2O mixture at 40 °C using catalytic amounts of Cu^{II} that was reduced in situ to Cu^{I} by an excess of sodium ascorbate. After overnight reaction, the addition was completed. This was proven by the disappearance of the azide peak (~ 2100 cm^{-1}) in the corresponding infrared spectrum.

The testosterone stationary phase was prepared using the same conditions as for the estradiol stationary phase, but now with 19-norethindrone in stead of 17-α-ethinyl estradiol. The structures of both stationary phases are given in **Figure V.13**.



Estradiol column Testosterone column

Figure V.13: Structures of the new stationary phases.

Column packing was performed using the slurry packing method. The set-up was shown in **Figure V.5**.

3.2.2.2 Chromatographic evaluation of the new columns

The stationary phases were not only interesting as media for the affinity screening, but in addition they offer also new selectivity for the analysis of complex mixtures. These columns were evaluated in depth in LC and SFC and compared to conventional columns for these techniques (Chapter VI).

3.2.2.3 Affinity LC study on the estradiol column

The columns were first evaluated using peptides with known affinity for 17-β-estradiol like the peptides of Tozzi (Chapter V.3.2.1). These peptides were analyzed on the estradiol column together with tetraglycine of which it was expected that it has no affinity for 17-β-estradiol, thus it will elute with the dead volume. The structures of the analyzed peptides are given in **Figure V.14**.

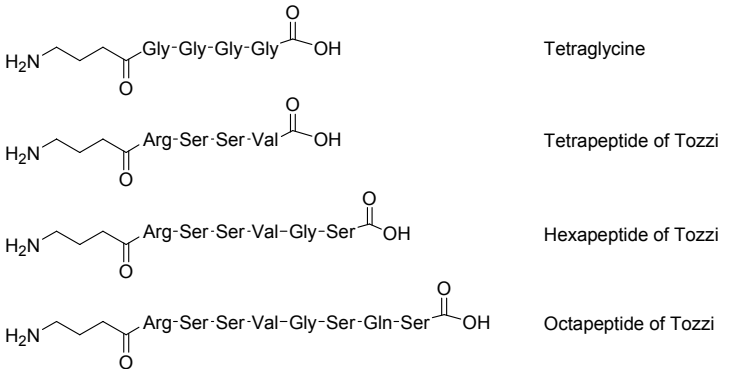


Figure V.14: Structures of the peptides used for the evaluation of the estradiol column.

The results of the analysis is presented in **Figure V.15**. The peaks are normalized to 100%.

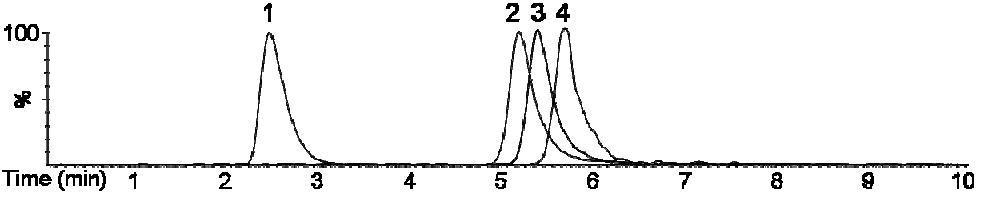


Figure V.15: Total ion chromatogram of the analysis of tetraglycine and the peptides of Tozzi on the estradiol column: Tetraglycine (1), Tozzi tetrapeptide (2), hexapeptide (3) and octapeptide.

All these peptides elute in the first part of the gradient, more specifically in pure water. Tetraglycine shows no affinity for estradiol and the retention times for the peptides are increasing from tetrapeptide to octapeptide, although it must be noted that the differences in retention for this analysis are rather small. When these data are compared to the dissociation constants determined by Tozzi and tabulated in **Table V.1**, a first discrepancy in the data is noticed.

Nevertheless, the difference in retention time between tetraglycine and the Tozzi peptides leads to the conclusion that the estradiol column can be used to recognize compounds with a certain affinity for estradiol. Hence, the synthesized library could be evaluated using this technique.

The synthesized receptors were first disconnected from the solid phase and then analyzed on the estradiol column. The general structure is shown **Figure V.16**.

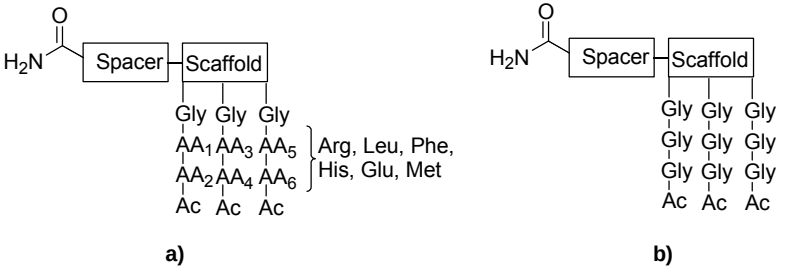


Figure V.16: General structure of the synthesized receptor (a) and the scaffold with glycines (b) for the analysis on the estradiol column.

In order to investigate the influence of the scaffold, a scaffold on which only glycines are attached, was also evaluated. This structure is given in **Figure V.16b**.

When the analysis is carried out isocratically at 100% 10mM NH_4OAc/NH_3, the synthesized receptors, do not elute, not even after 1h. Therefore, different eluting conditions were tested. In an attempt to disturb the hydrogen bounding, the pH was lowered until 3 but the receptor did still not elute. Then more drastic elution conditions were applied with a gradient to 100% MeOH in order to disturb the hydrophobic interactions. This eventually led to the elution of the receptor. The MeOH gradient was thus used for the screening of the library.

In the next chromatogram, the elution of the library members is shown together with that of tetraglycine, the tetrapeptide of Tozzi and the scaffold on which glycines are attached (**Figure V.17**). The peaks are all normalized to 100%.

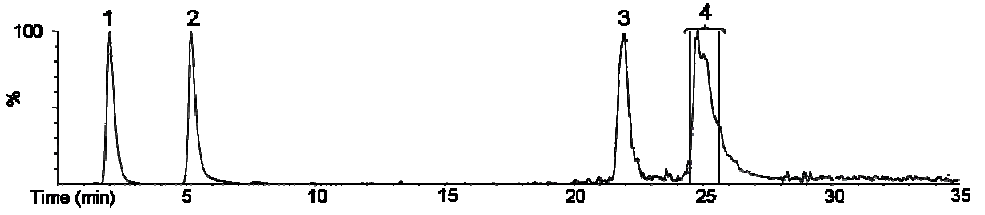


Figure V.17: Total ion chromatogram for the analysis of tetraglycine (1), Tozzi tetrapeptide (2), Scaffold with glycines (3) and the 120 members of the synthesized library (4) on the estradiol column.

Since the Tozzi tetrapeptide already elutes under aqueous conditions and pure methanol is necessary to elute the synthesized receptors, it can be concluded that the

synthesized receptors seems to have much more affinity for estradiol. Furthermore, the affinity of the synthesized receptors is mostly dedicated to the presence of the tripodal scaffold, because the difference in retention time between the scaffold with glycines and the library members is small. All the synthesized receptors have a retention time beween 24.61 and 25.53 min, so the different combinations of the amino acids seem to have a limited influence on the affinity.

3.2.2.4 Selectivity LC study on the testosterone column

Selectivity of the synthesized receptors was evaluated using the same analytical conditions as for the analyses on the estradiol column, but now on the testosterone column. The structure of this stationary phase was given in **Figure V.13**.

The library of synthesized receptors was designed to obtain no or weak affinity towards non-estrogenic compounds, so low retention should be obtained for the synthesized receptors on the testosterone column. The results are shown in **Figure V.18**.

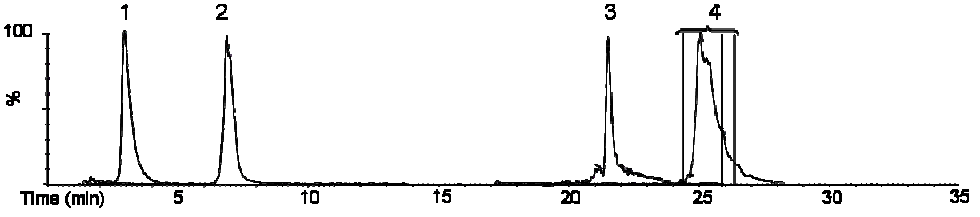


Figure V.18: Total ion chromatogram for the analysis of tetraglycine (1), Tozzi tetrapeptide (2), Scaffold with glycines (3) and the 120 members of the synthesized library (4) on the testosterone column.

When this chromatogram is compared to that for the analysis on the estradiol column (**Figure V.17**), it can be seen that there is almost no difference in the retention times. For the library members, this would indicate that there is a lack of selectivity. According to the results of Tozzi, which are demonstrated in **Table V.1**, the tetrapeptide would be more selective for estradiol. Consequently, the retention time on the estradiol column should be higher than on the testosterone column. Unfortunately, the retention times are similar, thus leading to the conclusion that the

tetrapeptide is not selective for estradiol. The results obtained by Tozzi et al. are therefore questionable.

3.2.2.5 Affinity or partition chromatography? A competition experiment

So far, it was assumed that the difference in retention time was the result of a difference in affinity for estradiol or testosterone. The type of separation mechanism was not investigated.

Since retention of a compound in affinity chromatography is caused by complex formation between the immobilized receptor and the free ligand in the mobile phase, the separation mechanism can be evaluated by adding the immobilized receptor also in the mobile phase. Complex formation will then also occur in the mobile phase, leading to a decrease in retention time of the ligand. In this contribution estradiol is immobilized on the stationary phase and the synthesized receptors are present in the mobile phase. By adding 17-β-estradiol to the mobile phase, the retention time of the synthesized receptors should decrease.

The best results were obtained when the amount of 17-β-estradiol in the mobile phase is the same as on the stationary phase. In order to limit the amount of 17-β-estradiol in the mobile phase, a capillary column (50 mm L x 0.25 mm ID, 5 µm d_p) was packed with the estradiol stationary phase.

The total amount of 17-β-estradiol in the capillary column is approximately 9 µmol. The synthesized receptor used for this experiment is shown in **Figure V.19**.

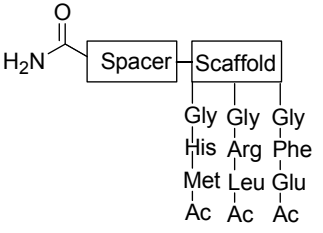


Figure V.19: Structure of the synthesized receptor used for the competition experiment.

First the synthesized receptor was analyzed without 17-β-estradiol in the background (**Figure V.20 a**). Next, approximately 3.7 mM (1000 ppm) 17-β-estradiol

was added to the mobile phase and the synthesized receptor was again analyzed (**Figure V.20 b**). Tetraglycine was also added in both analyses.

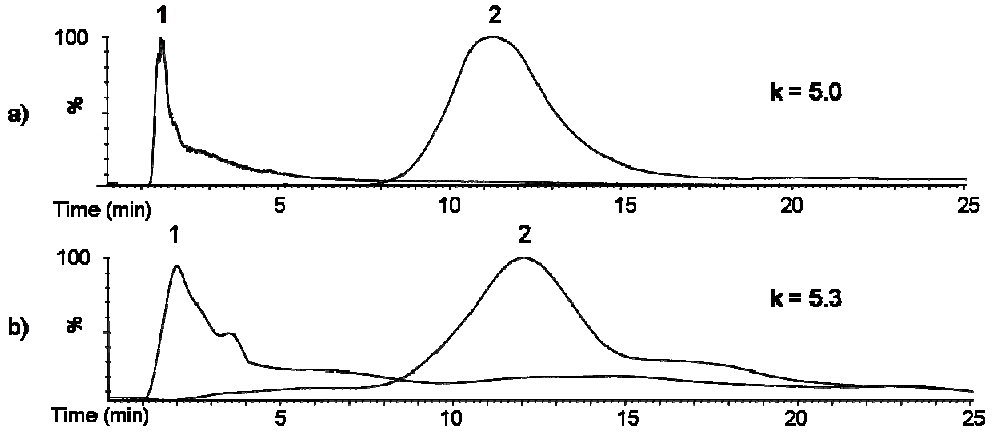


Figure V.20: Competition experiment: analysis of tetraglycine (1) and a synthesized receptor (2) on the estradiol capillary column without (a) and with (b) 17-β-estradiol in the background.

The retention factors for these two analyses were only slightly different. This leads to the conclusion, that the separation mechanism is mostly based on partition and not on affinity. Therefore, this technique could not be used for the screening of the library and another screening technique had to be developed.

3.2.3 Solid phase extraction

3.2.3.1 Evaluation of the backbone material

The synthesis of the artificial receptors was carried out on Tentagel-NH_2. This resin consists of a polystyrene framework that is cross-linked with 1 to 2 % divinylbenzene. Polyethylene glycol chains are grafted onto this network, rendering the resin compatible with polar solvents like water and thus making the resin more suitable for solid phase peptide synthesis [9]. The structure is given in **Figure V.21.**

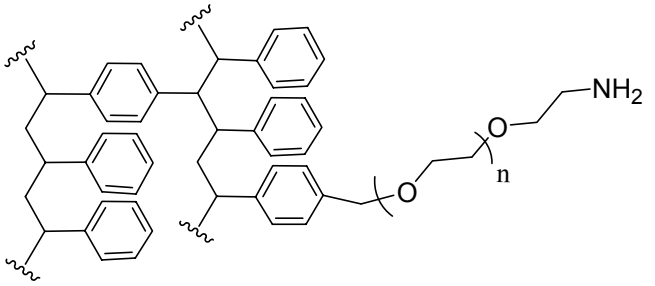


Figure V.21: Structure of Tentagel

The synthesized receptors bound to Tentagel were evaluated using the SPE procedure. However, it is possible that the EDCs are retained both by the Tentagel backbone and the synthesized receptor. In order to determine the contribution of Tentagel to the extraction efficiency, the affinity for Tentagel as such was also evaluated using the same procedure.

Figure V.22 shows the chromatogram of the water effluent of an empty SPE cartridge and of the water effluent after loading the SPE cartridge with Tentagel.

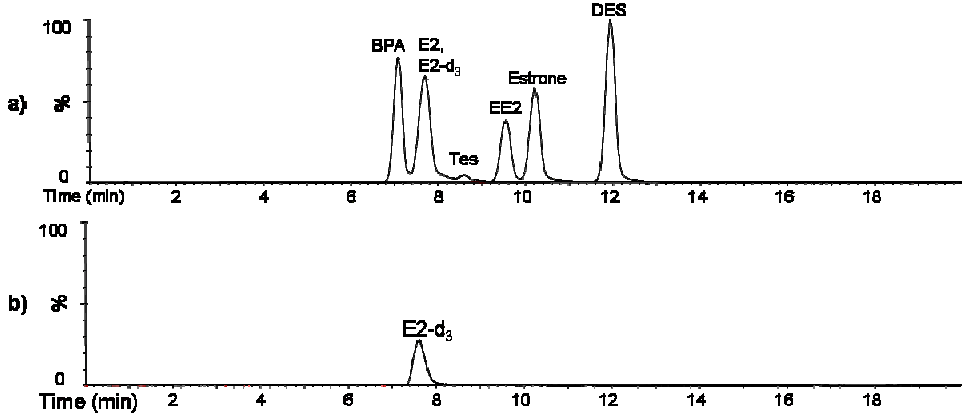


Figure V.22: Total ion chromatogram of the water effluent of an empty SPE cartridge (a) and after the SPE extraction with Tentagel (b).

As can be seen from this figure, Tentagel is able to almost completely extract the original EDCs. Only the internal standard E2-d_3 is present in the total ion chromatogram. As a consequence, it is impossible to evaluate the affinity of the synthesized receptors attached to Tentagel. Note that the area of E2-d_3 in the top

chromatogram is almost twice as high as in the lower chromatogram due to overlap of E2 and E2-d_3 in the top chromatogram. The results are also presented in **Figure V.24** as the relative amount of EDCs on Tentagel-NH_2.

The easiest solution to this problem is to change the solid phase used for the solid phase peptide synthesis. Crucial properties include physical and chemical stability as well as good swelling of the resin in solvents used for peptide synthesis. Gel type resins posses all of these qualities and are the most used in solid phase peptide synthesis. Some frequently used resins besides Tentagel-NH_2 are Merrifield, PEGA and Clear. Their structures are given in **Figure V.21** and **Figure V.23**.

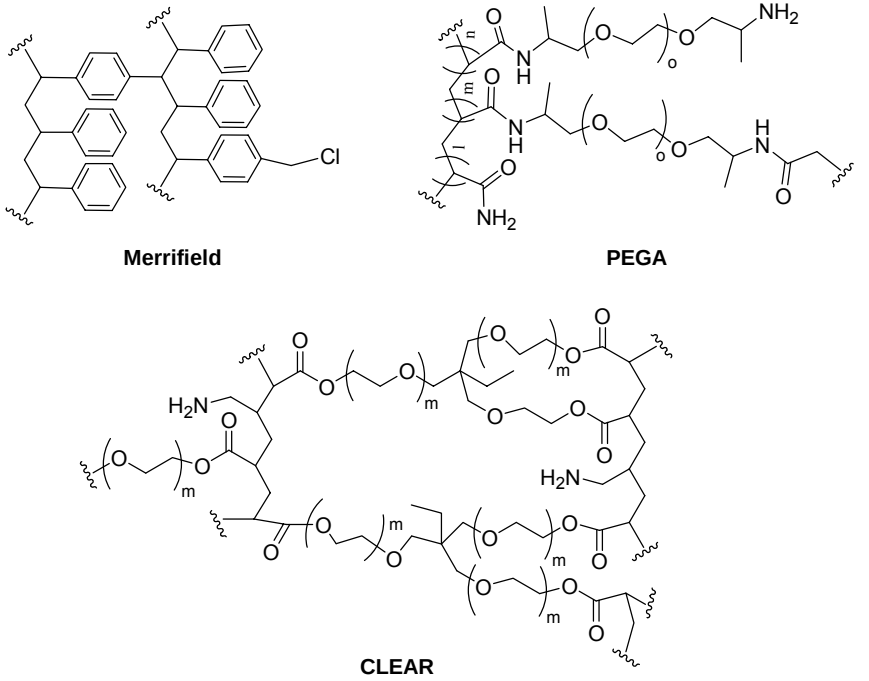


Figure V.23: Structures of other commercially available resins: Merrifield, PEGA and CLEAR

Merrifield consists, like Tentagel, of a polystyrene framework that is cross-linked with 1 to 2% divinylbenzene. Since Merrifield has no polyethyleneglycol chains grafted onto this network, the swelling capabilities in polar solvents are limited [9].

PEGA and CLEAR are resins that do not consist of a large hydrophobic core. PEGA is based on acrylamide monomers cross-linked with polyethylene glycol

chains, resulting in a very polar resin that has good swelling properties. Due to the presence of the amide bonds, a peptide like environment is created which is thought to be beneficial for peptide synthesis [10]. CLEAR consists of ester linkages with a high degree of cross-linking and a high percentage of polyethylene glycol chains. Consequently, the polar resin shows a high degree of swelling in especially polar solvents including water [11]. These three resins were also evaluated with the SPE procedure like Tentagel-NH_2. The results are expressed as the relative amount of EDCs that are trapped on the resins and are shown in **Figure V.24.** All the resins are tested in triplicate. The uncertainty of these measurements is determined as two times the standard deviation and is illustrated in this figure as error flags.

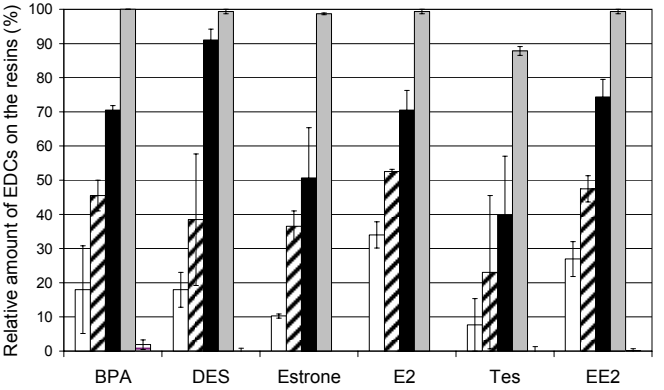


Figure V.24: Relative amount of EDCs trapped on the resins after SPE extraction with Merrifield (☐), Clear (▨), PEGA (■), Tentagel-NH_2 (▩) and Aminopropyl silicagel (〓).

As can be seen from this figure, Tentagel extracts more then 90% of the EDCs, while Merrifield only extracts between 8% (Tes) and 35% (E2) of the EDCs. This is probably caused by the limited swelling of Merrifield in aqueous media. Hence, the EDCs can not penetrate the core and interact with the bulk of the polymer. The extraction capability of Clear and PEGA for the EDCs is intermediate between Merrifield and Tentagel.

From these results, it could be concluded that the best resin for the solid phase peptide synthesis and subsequent SPE screening would be Merrifield. Unfortunately, its limited swelling in polar solvents causes dramatic problems for the synthesis and

can therefore not be used as an alternative to Tentagel. Consequently, the next resin of choice becomes Clear resin. The synthesis has been carried out on this resin, but was never successful. PEGA was now the only possible resin left for the synthesis. Not only, this resin extracts already a high amount of the EDCs (**Figure V.24**), but in addition the material is difficult to handle (statically charged). PEGA is provided as a suspension in MeOH but upon drying, the resin becomes impossible to handle.

Since all these resins have their specific problems, aminopropyl silicagel was evaluated. This material is frequently used as column material in chromatography. It has never been used as solid phase material for solid phase peptide synthesis. First, the extraction capability of aminopropyl silicagel itself was investigated using the SPE procedure. The results are expressed as the relative amount of EDCs that were extracted with the SPE procedure and are also shown in **Figure V.24**. Aminopropyl silicagel hardly extracts the EDCs and becomes therefore the ideal material for the synthesis and screening of the library. One of the library members was synthesized on aminopropyl silicagel. Not only was the overall purity of the library member less than on Tentagel but more important, deprotection of the side chains of the amino acids could not be brought to completion.

It could therefore be concluded that the synthesis is only successful when carried out on Tentagel. The library was consequently synthesized on Tentagel. In contrast, the SPE procedure was most successful when it is carried out on aminopropyl silicagel. This can be realised by performing the synthesis on Tentagel and afterwards disconnecting the library member from the Tentagel and re-connecting it to aminopropyl silicagel. This was a lot of experimental work and therefore it has only been performed for two library members.

3.2.3.2 Evaluation of the synthesized receptors

The SPE method was first evaluated with the tetrapeptide of Tozzi. The structure is given in **Figure V.25**. Afterwards, two of the 120 members of the synthesized library were connected to aminopropyl silicagel and evaluated using the SPE procedure. Their structures are also shown in **Figure V.25.**

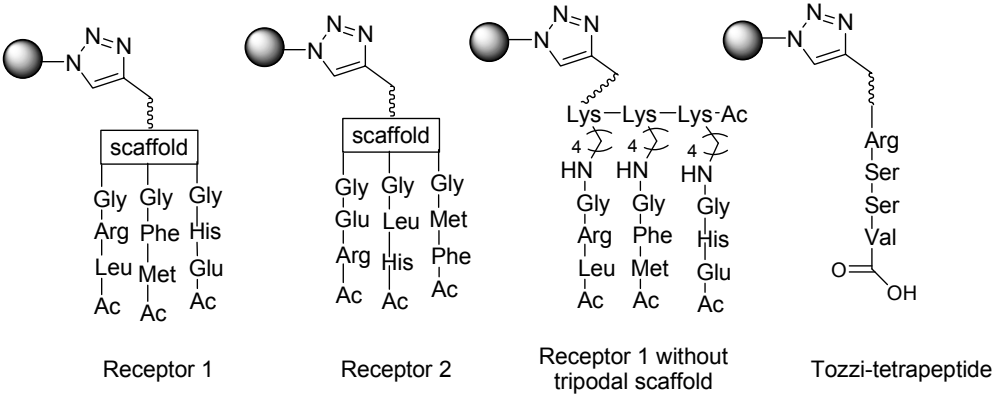


Figure V.25: Structures of two synthesized receptors, one of the receptors without the tripodal scaffold and the tetrapeptide of Tozzi, all attached to aminopropyl silicagel.

In order to investigate the contribution of the tripodal scaffold to the observed interaction with the EDCs, the amino acid sequence of receptor 1 was attached to aminopropyl silicagel without the scaffold and evaluated using the same SPE procedure. The data expressed as the relative amount of EDCs that have been trapped on the beads are depicted in **Figure V.26**. The synthesized resins are corrected for their loading and normalized for their weight.

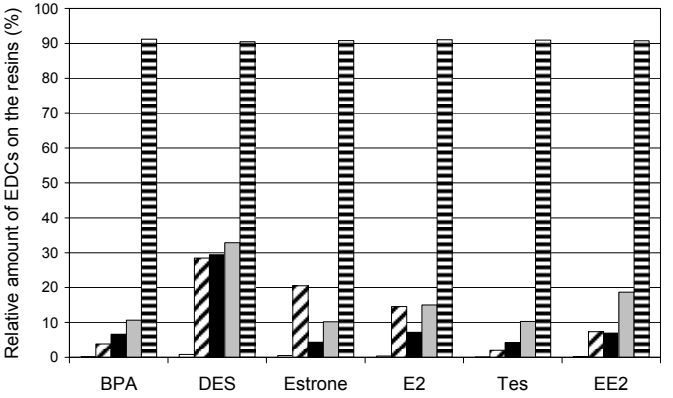


Figure V.26: Relative amount of EDCs trapped on the beads after SPE procedure with the tetrapeptide of Tozzi (□), Receptor 1 without tripodal scaffold (▨), Receptor 1 (■), Receptor 2 (▩) and Oasis HLB® (▤).

From this figure it is clear that receptor 1 and 2 show an increased affinity towards estrogenic compounds when compared to the unmodified silica of which the results are shown in **Figure V.24**. More surprising is that the receptors retain the various EDCs better than the Tozzi-tetrapeptide, which does show any affinity whatsoever. It is more likely that these effects are the result of non-specific interactions and that there is no real complex formation between the receptors and the various ligands. Indeed, when looking at the receptor without scaffold, but with the same amino acid build up as receptor 1, there is no significant difference between the two materials. This is due to a combined effect of non-specific hydrogen and Van der Waals bonding between the estrogenic compounds and the amino acids of the compounds. Another disappointing conclusion is that no selectivity is observed. The extraction efficiency of the two receptors towards 17-β-estradiol and testosterone are not significantly different.

The properties of the synthesized materials were compared with the commercially available SPE cartridge Oasis HLB®. This SPE material is hydrophilic-lipophilic balanced [12]. It has been successfully applied for the extraction of EDCs in aqueous samples by Lopez de Alda et al. [13]. In this contribution, 10 mg of Oasis HLB® was evaluated with the SPE procedure and the results are shown in **Figure V.26**. This SPE material is able to extract more then 90% of the EDCs, which is much better than the extraction efficiencies of the synthesized receptors.

3.2.3.3 Analysis of a real water sample

When a complex matrix is analysed, the extraction efficiencies of Oasis HLB® are expected to be lower in comparison to the analysis of a clean aqueous sample, due to the non-specific interactions, leading to intensive competition between the analytes in the aqueous sample.

Both Oasis HLB and receptor 2 were used for SPE extraction of hospital effluent water of the University hospital of Ghent spiked with EDCs. The results are expressed as relative amount of EDCs trapped on the beads and are presented in **Figure V.27**.

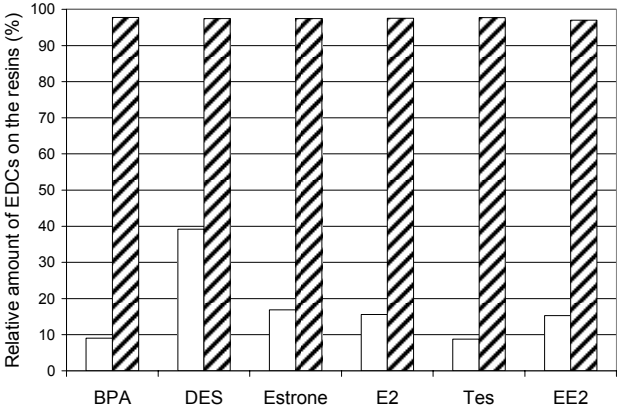


Figure V.27: Relative amount of EDCs trapped on the beads after SPE extraction of hospital water spiked with EDCs with Receptor 2 (□) and Oasis HLB® (▨).

Even for the analysis of a real water sample, the extraction performance of Oasis HLB® is much better than that of the synthesized receptor. Consequently, Oasis HLB® can be used for the analysis of EDCs from aqueous samples.

3.2.4 Conclusion

A library of possible mimics of the estrogen receptor was prepared. The affinity of the members towards 17-β-estradiol was evaluated using two different orthogonal screening techniques. The first one is based on affinity liquid chromatography. Affinity columns were prepared with estradiol or testosterone bound to the stationary phase. Both columns were first evaluated using peptides with known of affinity for 17-β-estradiol. These are the peptides prepared by Tozzi et al.. Afterwards, the library was evaluated on both columns. The synthesized receptors show affinity, but no selectivity towards 17-β-estradiol. A competition experiment, where 17-β-estradiol was present in the mobile phase was carried out to investigate the separation mechanism. It was concluded that the separation was based mostly on partition and not on affinity. Consequently, the estradiol and testosterone column were not suitable as screening technique for the synthesized library.

The second screening technique was based on solid phase extraction. Since the solid phase used for the synthesis extracts all EDCs, another solid phase should be used for the SPE procedure. The synthesis was carried out on Tentagel and afterwards the library members were disconnected and immobilized on aminopropyl silicagel. The extraction efficiency of the tetrapeptide of Tozzi was compared to that of two library members and to commercially available Oasis HLB® SPE material.

One of the synthesized receptors and Oasis HLB® were used for the analysis of hospital effluent spiked with EDCs.

It can be said that though materials have been made that can withhold EDCs from an aqueous sample, a comparison with a well-known SPE cartridges like Oasis showed that the amounts trapped are not sufficient to justify their use as a clean-up procedure before actual chemical analysis.

4 References

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CHAPTER VI
EVALUATION OF THE ESTRADIOL AND TESTOSTERONE STATIONARY PHASES IN HPLC AND SFC*

In the previous chapter, the principle of 'click' chemistry was used to immobilize testosterone and estradiol on aminopropyl silicagel.

The chromatographic performance of these new stationary phases was evaluated both in reversed LC and supercritical fluid chromatography (SFC) using different mixtures. Their selectivity was compared to the commonly used columns in LC and SFC, namely octadecyl silicagel (C18) and 2-ethylpyridine, respectively.

* To be published as:
'Click chemistry used as an efficient strategy for the manufacturing of dedicated stationary phases for LC and SFC'
E. Van Hoeck, S. Van der Plas, M. Dunkle, F. Lynen, A. Madder, P. Sandra, in preparation

1 Introduction

Nowadays, reversed phase LC on a silica based C_{18} stationary phase is the most popular and efficient chromatographic technique. However, not all separation problems can be resolved using this stationary phase, especially for the separation of stereoisomers [1,2] positional isomers [3], very polar compounds [4] and the separation of complex samples in the fields of metabolomics, drug discovery and natural product research [5]. Therefore, the development of new versatile and tailor made separation media is desirable. One approach to achieve this goal is to modify the C18 stationary phase with more polar groups. However, C18 remains the major domain in these modified C18 ligands and consequently, the changes in separation selectivity are limited [6].

Another approach is to immobilize new molecules with the desired chemistry on a silica support. Pesek et al. used this strategy and prepared a cholesterol bound stationary phase. The hydrophobic/hydrophilic properties of cholesterol allow this material to be used for both reversed phase and aqueous normal phase separations [7]. Catabay et al. described the analysis of 1,4-benzodiazepines on a cholesteryl-10-undecenoate bonded stationary phase. It was found that the selectivity on this phase was remarkable different compared to the C18 column [8].

The reactions to immobilize functionalized molecules onto the solid support are usually traditional nucleophilic and electrophilic reactions. These conjugations involve reactive groups such as $–NH_2$, -COOH, -COCl, -CHO, etc. on the supports and the functionalized molecules [1,2,9]. Side reactions may occur that can result in reducing or even losing the function of the stationary phase. In addition, the reactive groups on the silica supports may not be fully converted which will reduce the surface concentration of the stationary phases. There is no doubt that the remnant reactive groups on the support will exhibit different retention mechanisms, thus affecting the separation chemistry [10]. The development of a robust, reliable immobilization method with high selectivity under mild conditions for preparation of functionalized HPLC packings remains a challenge in separation chemistry.

Click chemistry provides an ideal reactivity profile for this purpose. This type of reactions was already discussed in Chapter V.3.2.2.1. In short, the primary reaction of click chemistry is the copper(I) catalyzed [3+2] dipolar cycloaddition between organic azides and terminal alkynes described by Sharpless et al. [11] and demonstrated in **Figure V.11**. This reaction received more and more attention in many chemistry research fields, including combinatorial chemistry [12], material science [13], solid phase reactions [14] and surface modification [15]. Recently, Slater et al. reported the application of click chemistry on acrylate polymer beads, for the preparation of HPLC packing material [5]. Lummerstorfer et al. investigated the [3+2] reaction of azides immobilized on silica with acetylenes and it was found that all the azide groups were converted to 1,2,3-triazoles [5]. Guo et al, demonstrated the usage of click chemistry to immobilize different alkyne bearing molecules on silica. Before the reaction was carried out, azide groups were built in on silica beads by reaction of 3-azidopropyl triethoxysilane with silica [16]. Later on, the same group reported the synthesis of an oligo(ethyleneglycol) stationary phase using the same procedure [17].

In this work, 19-norethindrone and 17-α-ethinyl estradiol were immobilized on aminopropyl silicagel using click chemistry. The chromatographic performance of the columns was evaluated in reversed phase LC and SFC. The efficiency and selectivity were also compared to the commonly used columns in LC and SFC, namely octadecyl silica (C18) and 2-ethylpyridine, respectively.

2 Experimental

2.1 Chemicals

Neat certified endocrine disrupting standards were purchased from different sources. Desethylatrazine and sulfamethoxazole were purchased from Riedel de Haën (Seelze, Germany). Delmadinone acetate and alfason were supplied by Steraloids (Newport, Rhode Island, U.S.A). Propyl paraben, toluene, benzene, nortestosterone, 17-β-estradiol, ketoprofen, naproxen, fluoranthene, benzo(k)fluoranthene, benzo(g,h,i)perylene, indeno(1,2,3-cd)pyrene, ibuprofen, theophylline, theobromine, thymine, adenine, uracil, flurbiprofen, cortisone, prednisolone, cytosine, hypoxanthine, hydrocortisone, prednisolone, sulfamerazine, estriol and sulfaguanidine were purchased from Sigma-Aldrich (Bornem, Belgium). Stanozolol, chlorotestosterone acetate, testosterone propionate, metribuzin, cyanazine, prometryn, terbutryn, testosterone, medroxyprogesteron aceate, fenoprofen, sulfadimethoxine, sulfaquinoxaline, sulfamethizole, caffeine and benzo(a)pyrene were supplied by Fluka (Bornem, Belgium).

Water, methanol (MeOH), acetonitrile (ACN) and formic acid (all MS grade) were supplied by Biosolve (Valkenswaard, The Netherlands). Amonium acetate (NH_4OAc) and ammoniak (NH_3) were purchased from Sigma-Aldrich (Bornem, Belgium). The SFC mobile phase consisted of CO_2 (N45 quality), purchased from Air Liquide (Liege, Belgium).

2.2 Instrumentation

LC-MS analyses were carried out on an Alliance 2690 LC system equipped with an on-line degasser and an autosampler (Waters Milford, MA, USA).

The analyses were carried out on the home-made estradiol or testosterone column (150 mm L x 2.1 mm ID, 5 µm d_p) or a Luna-C18 column (150 mm L x 2.1 mm ID, 5 µm d_p) (Phenomenex, Torrance, CA, USA). The columns were thermostated at 30°C. Different gradients were applied for the analysis of the different mixtures. The

gradients are summarized under the corresponding chromatograms. The flow rate was 0.2 mL/min and the injection volume was 5 µL.

Ultraviolet detection was carried out using a Waters 2487 dual λ absorbance detector (Waters, Milford, MA, USA). Analyses were recorded at 210 and 230 nm.

Mass spectrometric detection was performed using a Quattro Micro system equipped with a Z-spray electrospray source (Micromass Manchester, UK). The mass spectrometer was used in the positive mode. The capillary voltage was set at 3 kV and the cone voltage at 30 V. Mass spectra were recorded in scan mode beween 135 and 430 m/z. Data acquisition, instrument control and data analysis were performed by Masslynx software (version 4.0, Micromass).

SFC-UV experiments were performed on a Berger SFC Minigram (Mettler-Toledo, AutoChem, Newark, DE, USA), equipped with a dual pump fluid control module (FCM-1100/1200), a heater control module (TCM-2250), a peak detector module (PDM-1250) and an autosampler (ALS-3100/3150). The analyses were run on the home-made estradiol and testosterone column or on a 2-ethylpyridine column (250 mm L x 4.6 mm ID, 3 µm d_p) (Princeton Chromatography, Cranbury, NJ, USA). Analyses were performed at constant flow rate 2.0 mL/min, at a temperature of 40 °C and at a constant outlet pressure of 100 bar. Methanol was added as organic modifier with a program from 5%, hold for 1 min, to 40% at a rate of 2.0%/min. UV Detection was carried out at λ = 254 nm. The injection volume was 5 µL.

3 Results and discussion

The immobilization of 19-norethindrone and 17-α-ethinyl estradiol on aminopropyl silicagel using click chemistry and the packing of the columns was previously described in Chapter V.3.2.2.1. The structures of the stationary phases are shown in **Figure VI.1**.

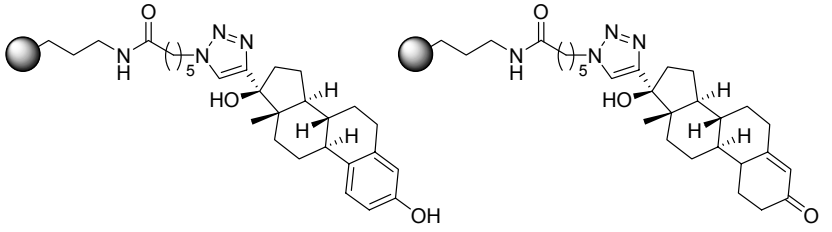

Figure VI.1: Structures of the new stationary phases.

3.1 Evaluation in reversed phase LC

The column performance was evaluated using different mixtures. A first mixture contained propyl paraben and toluene. This mixture was used to evaluate the efficiency and hydrophobicity of the stationary phases. **Figure VI.2** shows the resulting chromatograms for a C18 and testosterone column.

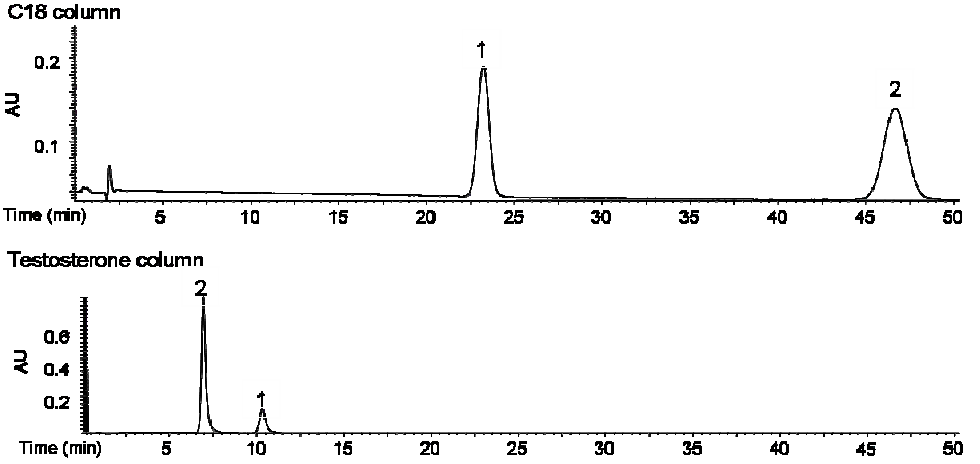

Figure VI.2: Analysis of propyl paraben (1) and toluene (2) on C18 and testosterone column. Conditions: Isocratic elution: 70:30 (H_2O,10mM NH_4OAc/NH_3:ACN) @ 0.2 mL/min. UV detection @ 210 nm.

The testosterone phase is less hydrophobic compared to a C18 phase. Concerning the efficiency, reduced plate heights (h) were 3.8 and 3.4 for the estradiol and testosterone phase, respectively. This is less efficient compared to C18 (h = 2.2), which is typical for polar interactions as observed in normal phase LC. The reversed elution order for propyl paraben and toluene in a shorter analysis time emphasizes this behaviour.

A more difficult mixture to separate is a steroid mixture. The analysis on the C18 and the testosterone column are shown in **Figure VI.3**.

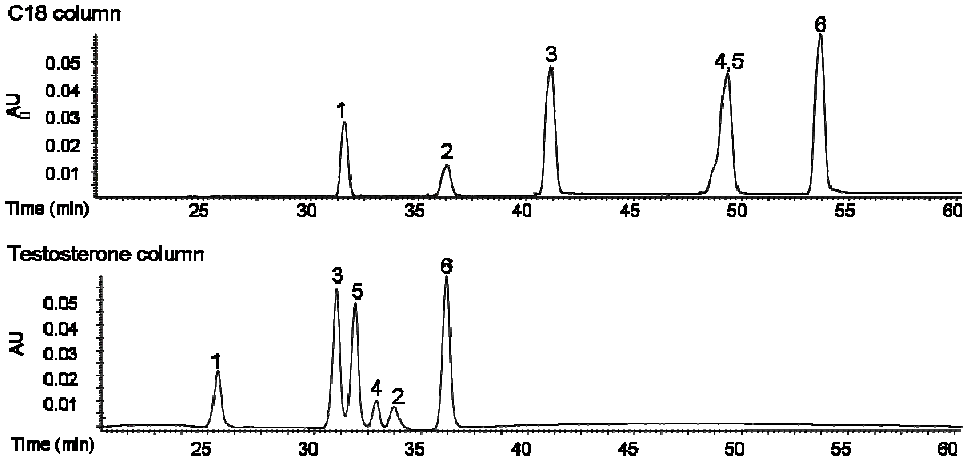

Figure VI.3: Analysis of a steroid mixture containing alfason (1), stanozolol (2), delmadinone acetate (3), chlorotestosterone acetate (4), testosterone propionate (5) and nortestosterone (6) on C18 and testosterone column.
Conditions: Gradient elution: 0-60 min: from 100% H_2O to 100% ACN @ 0.2 mL/min.
UV detection @ 210 nm.

The testosterone stationary phase is more selective for steroids as it separates chlorotestosterone acetate and testosterone propionate while they co-elute on the C18 stationary phase. This can be explained by the like-likes-like principle of chromatography.

Finally, a very complex mixture was chosen to illustrate the separation capabilities of the two new stationary phases compared to C18. The resulting chromatograms are given in **Figure VI.4**.

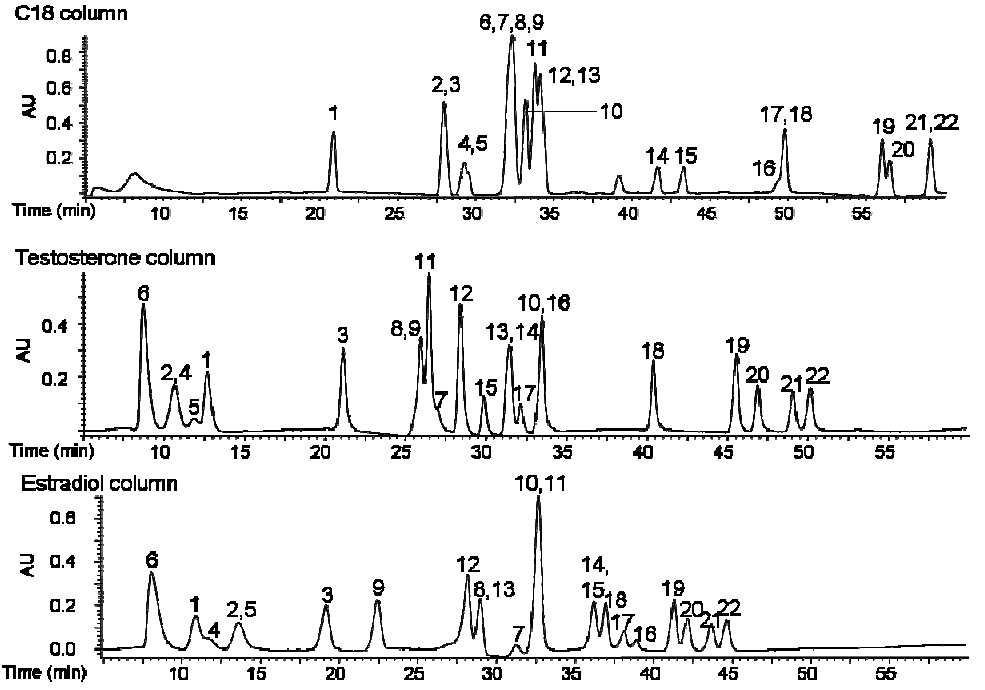

Figure VI.4: Analysis of a complex mixture on C18, testosterone and estradiol columns. The used compounds are: desethylatrazine (1), metribuzin (2), cyanazine (3), prometryn (4), terbutryn (5), benzene (6), stanozolol (7), alfason (8), propyl paraben (9), 17-β-estradiol (10), testosterone (11), ketoprofen (12), naproxen (13), delmadinone acetate (14), medroxyprogesterone acetate (15), chlorotestosterone acetate (17), fluoranthene (18), benzo(k)fluoranthene (19), benzo(a)pyrene (20), benzo(g,h,i)perylene (21), indeno(1,2,3-cd)pyrene (22).

Conditions: Mobile phase: H_2O with 0.1% HCOOH (A) and ACN (B)

Gradient: 0-60 min: from 0% B to 100% B @ 0.2 mL/min

UV detection @ 210 nm and 230nm.

When comparing the elution order of the complex mixture on the three columns, it is clear, that the interaction with the stationary phase in each case is very different, leading to a completely different elution profile. For example, cyanazine and metribuzin co-elute on C18, but the difference in retention time for these two compounds, when analyzed on the steroid columns, is more than 15 min. Another example is the analysis of PAHs. When they are analyzed on the C18 column,

benzo(k)fluoranthene (19) co-elutes with benzo(a)pyrene (20) and benzo(g,h,i)perylene (21) co-elutes with indeno(1,2,3-cd)pyrene.(22). When the steroid columns are used, all these PAHs are baseline separated.

In conclusion, the selectivity of these new types of stationary phase is completely different compared to C18 and this can be exploited for the separation of complex samples.

3.2 Evaluation in SFC

The testosterone column was also used as stationary phase in SFC. The obtained selectivity was compared to a commonly used stationary phase in SFC, namely 2-ethylpyridine. The analyses were carried out by Melissa Dunkle of our research group. The resulting chromatograms are shown in **Figure VI.5**.

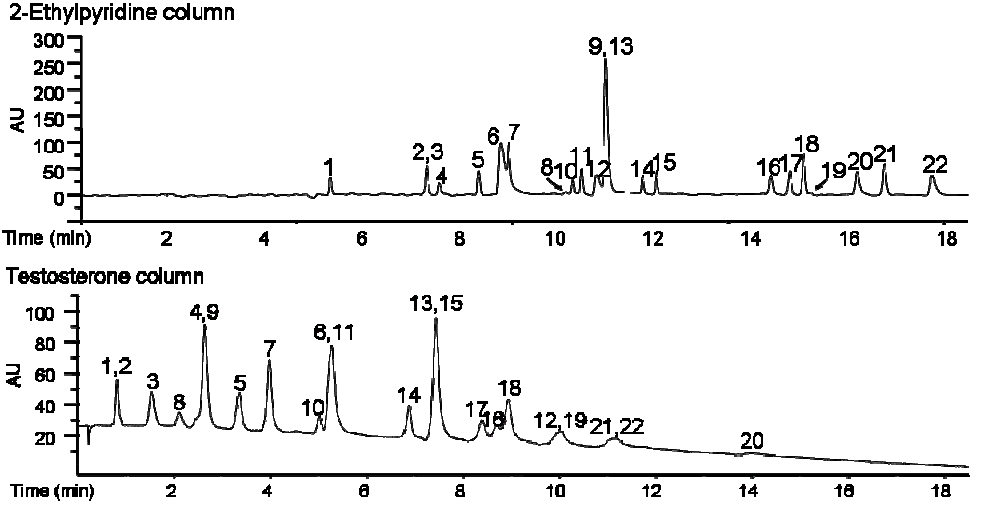

Figure VI.5: Analysis of a complex mixture by SFC: caffeine (1), ibuprofen (2), theophylline (3), theobromine (4), thymine (5), adenine, (6), uracil (7), fenoprofen (8), flurbiprofen (9), cortisone (10), prednisone (11), cytosine (12), hypoxanthine (13), hydrocortisone (14), prednisolone (15), sulfamerazine (16), sulfamethoxazole (17), sulfadimethioxime (18), estriol (19), sulfaguanidine (20), sulfaquinoxaline (21), sulfamethizole (22)

The testosterone column has some potential to be used in SFC and the selectivity is completely different compared to the 2-ethylpyridine column. In depth evaluation is presently carried out in our laboratory.

4 Conclusion

Two new stationary phases were prepared by immobilizing the steroids 19-norethindrone and 17-α-ethinyl estradiol on aminopropyl silicagel using click chemistry. The efficiency and selectivity of the columns was evaluated in reversed phase LC and SFC with different mixtures.

The results show that the steroid columns have a completely different selectivity compared to C18 for reversed phase LC and to 2-ethylpyridine for SFC.

Click chemistry is fast, simple and easy to prepare dedicated stationary phases.

This short study was the initiation to intensify research activities in our research group on the synthesis of several stationary phases based on click chemistry.

5 References

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GENERAL CONCLUSION

The determination of endocrine disrupting chemicals in aqueous samples is very challenging. Not only are the EDCs chemically very heterogeneous, but they cause adverse effects at concentration levels as low as 1 ng/L. In addition, the environmental matrix is very complex. Therefore, sample clean-up en pre-concentration of the sample is necessary prior to analysis.

In the framework of this thesis, attempts were made to develop a multi-residue method that is able to identify and quantify the EDCs at the very low levels at which they are present in aqueous samples. Stir bar sorptive extraction (SBSE) was used as sample preparation technique. Different desorption modes, i.e. thermal desorption in a dedicated desorber, in a classical split/splitless inlet equipped with a flip-top device and liquid desorption were compared for the analysis of pyrethroids in water samples. Sensitivity was the highest for thermal desorption in a dedicated thermal desorption unit. As a consequence, this type of desorption was chosen for the multi-residue method for the determination of EDCs. This multi-residue method is a multi-shot SBSE-TD-GC-MS method. It allows simultaneous analysis of different classes of EDCs and pharmaceuticals. Four aliquots of 10 mL water were taken for stir bar sorptive extraction (SBSE) and they were treated in the following way. In sample one, *in-situ* derivatization is performed with acetic acid anhydride to improve extraction efficiencies and chromatographic analysis for phenolic compounds. For the same reasons, for amines and acids, aliquot two is treated with ethyl chloroformate and aliquot three with tetraethylborate for organitin compounds. To sample four methanol is added to destroy wall adsorption for apolar solutes. After SBSE, the four stir bars together with a plug of glass wool impregnated with bis(trimethylylsilyl)trifluoroacetamide (BSTFA) to derivatize hydroxyl functionalities, were introduced in the same thermal desorption tube, heat desorbed and analysed simultaneously by capillary GC-MS. The limits of detection that can be achieved using the optimized multi-residue methods are in the range of 0.01 and 22 ng/L. The performance of the method was illustrated with the analysis of some real

water samples. Ketoprofen, bisphenol A, 17-β-estradiol, propofol, tributyltinchloride and β-sitosterol were successfully detected in hospital effluent of the university hospital in Ghent, Belgium and in surface water collected from a water treatment plant in Torino, Italy.

Since the enrichment of very polar analytes with SBSE is still limited, an attempt was made to overcome this drawback. Two different approaches were evaluated. In the first strategy, a novel sorptive extraction technique was developed, namely silicone membrane sorptive extraction (SMSE). It was first optimized for the determination of atrazine and its polar metabolites desethylatrazine, desisopropylatrazine and desethyldesisopropylatrazine in aqueous samples. Due to the presence of an organic solvent inside the PDMS tube, the extraction efficiency is significantly increased, leading to a higher sensitivity. The limited solubility of ethyl acetate in water causes the need for dilution of the extract with water before LC-MS analysis. GC-MS in combination with large volume injection is therefore more preferable, leading to higher sensitivities. A DB-17MS column is preferred over a DB-WAX column, since the latter showed peak distortion of the analytes in combination with large volume injection. The SMSE-GC (DB-17MS)-MS method is able to screen for atrazine, desethylatrazine, desisopropylatrazine and desethyldesisopropylatrazine at low ng/L (ppt) levels. The applicability of the method was then evaluated for a complex mixture of EDCs and pharmaceuticals with a wide variety in polarity. It could be concluded, that this technique is only beneficial for very polar analytes (log $K_{o/w} < 2$). When less polar analytes are determined, they are preferable present in the PDMS leading to lower recoveries in the ethyl acetate extract. Consequently, this type of sample preparation can not be used in a multi-residue method.

A second strategy to improve the extraction efficiency of polar analytes employed monolithic material as extraction medium. The monolithic material was prepared using an in-situ polymerization of acrylamide, 4-vinylpyridine and N,N'-methylene bisacrylamide. After the synthesis, the material was characterized using SEM analysis, FT-IR spectroscopy and TGA analysis. In order to evaluate the extraction capabilities of the monolithic material, it was used for the analysis of a complex

mixture of EDCs and pharmaceutical. Due to lack of robustness of the monolithic material when it is used as stirring bar in aqueous samples, the results were very disappointing. Consequently, the monoliths were evaluated in the headspace, more specifically the static headspace of coffee. The results were then compared to those obtained with conventional PDMS. The extraction efficiency of the monolith was less compared to PDMS. This is ascribed to the adsorption mechanism rather than the sorptive mechanism occurring on PDMS. Furthermore, the degradation products of the monolith disturb the background, thereby complicating identification and quantification.

In conclusion, none of the two proposed strategies were able to fill the gap of extraction of polar analytes in combination with a multi-residue method.

At last, attempts were made to develop a more selective sample preparation procedure by synthesizing a library of artificial mimics of the estrogen receptor. The affinity of the members towards 17-β-estradiol was evaluated using two different orthogonal screening techniques. The first one is based on affinity liquid chromatography. Affinity columns were prepared with estradiol or testosterone. The library was evaluated on both columns. The synthesized receptors show affinity, but no selectivity towards 17-β-estradiol. A competition experiment, where 17-β-estradiol was present in the mobile phase was carried out to investigate the separation mechanism. It was concluded that the separation was based mostly on partition and not on affinity. Consequently, the estradiol and testosterone columns were not suitable as screening technique for the synthesized library.

The second screening technique was based on solid phase extraction. Since the solid phase used for the synthesis extracts all EDCs, another solid phase should be used for the SPE procedure. The synthesis was carried out on Tentagel and afterwards the library members were disconnected and immobilized on aminopropyl silicagel. The extraction efficiency of two library members was compared to commercially available Oasis HLB® SPE material for spiked laboratory water and spiked hospital effluent. It can be said that though materials have been made that can withhold EDCs from an aqueous sample, comparison with a well-known SPE material like Oasis

HLB® showed that the amounts trapped are not sufficient to justify their use as a clean-up procedure before actual chemical analysis.

The new stationary phases that were used in the affinity LC study were also chromatographically evaluated on reversed phase LC and SFC with different mixtures. The results show that the steroid columns have a completely different selectivity compared to C18 for reversed phase LC and 2-ethylpyridine for SFC. In conclusion, click chemistry is fast, simple and easy to prepare dedicated stationary phases.

Although this work merely tips the iceberg with regard to providing total insight in the analysis of EDCs in aqueous samples, it aims to present the reader a broad overview of the different analytical techniques that can be used, their corresponding shortcomings and possible solutions. Furthermore, this work aims at inciting other investigators and governmental bodies to continue the research in this area, as large deficits remain especially with the regard to environmental and public safety.

SUMMARY

In the last decade, the increasing distribution of endocrine disrupting chemicals (EDCs) in the environment has been a worldwide concern. This anxiety is caused by the adverse effect of these pollutants on the endocrine system of humans and wildlife, even at levels as low as 1 ng/L. While the influence on the reproductive system of several animals has been thoroughly documented, the effects on human health are still the subject of intense debate.

Chapter 1 gives a brief overview of the mechanism of endocrine disruption and of the effects on wildlife and humans. Furthermore, the possible sources of exposure of humans and wildlife to EDCs are illustrated, together with the different approaches for the detection of EDCs in aqueous samples.

In *Chapter 2*, the determination of EDCs in aqueous samples is discussed. Not only are the EDCs chemically very heterogeneous, but the environmental matrix in which they are present is very complex. Furthermore, the developed methods should be able to detect the EDCs at the very low concentrations at which they are present in water samples. Therefore, sample clean-up and pre-concentration form the key step prior to analysis. An overview is presented of the most common sample preparation methods for aqueous samples. Their use for the determination of EDCs is illustrated.

Chapter 3 describes the development of a multi-residue method for the determination of EDCs in aqueous samples. Stir bar sorptive extraction (SBSE) was first applied to the enrichment of pyrethroids in water samples to evaluate the different desorption techniques. Thermal desorption (TD) was performed in a classical split-splitless inlet equipped with a flip-top sealing system and in a dedicated thermal desorption unit. These two thermal desorption methods were compared to liquid desorption with ethyl acetate. Several parameters that influence extraction and desorption efficiency were evaluated. Sensitivity was the highest for thermal desorption in a dedicated thermal desorption unit. Therefore, this procedure was used for the development of a multi-residue method for EDCs in aqueous samples. Four different sample preparation procedures carried out in parallel on four aliquots of the

same water sample were performed. Three derivatisation reactions specific to phenolic compounds, amines and acids, and organometallic compounds, respectively, were applied to three sample aliquots, while compounds with a log $K_{o/w}$ compatible with PDMS and not requiring derivatization were sampled in the fourth aliquot. In-tube silylation was carried out with BSTFA. The resulting stir bars are introduced in the same thermal desorption tube, heat desorbed and analysed simultaneously by capillary GC-MS. The figures of merit of the method were evaluated with an EDC model mixture. The performance of the method is illustrated with the analysis of some real water samples.

The extraction of polar analytes in aqueous samples is very difficult. In *Chapter 4*, two different strategies were evaluated in order to improve the extraction efficiency of these solutes. The first strategy encompasses a novel sorptive extraction technique, namely silicone membrane sorptive extraction (SMSE). A PDMS tube is filled with an organic solvent and placed in the aqueous sample for extraction. Afterwards, the organic solvent in the PDMS tube is analysed by large volume injection GC-MS or LC-MS. The extraction was optimized for the determination of atrazine and its metabolites. Afterwards, the applicability of SMSE was evaluated for a complex mixture of EDCs and pharmaceuticals. In the second part of this chapter a new stir bar extraction material based on monoliths was prepared. The extraction capabilities of this material were evaluated for the static headspace analysis of coffee and compared to conventional extraction with PDMS. It is impossible to use this material as effective as SBSE due to the lack of robustness of this material.

In *Chapter 5* a library of possible mimics of the estrogen receptor was prepared. The affinity of the members towards 17-β-estradiol was evaluated using two different screening techniques. The first one is based on affinity liquid chromatography. An affinity column was prepared where 17-α-ethinyl estradiol or 19-norethindrone was bound to the stationary phase. The second screening technique is based on solid phase extraction. The extraction efficiency of two library members was compared to that of a commercially available SPE material Oasis HLB® for laboratory water and hospital effluent, both spiked with EDCs.

Although very promising results were expected, based on literature, the presented techniques were unsuccesfull.

Chapter 6 discusses the chromatographic performance of the estradiol and testosterone stationary phase prepared in the previous chapter for the affinity LC study. These stationary phases were evaluated both in reversed LC and supercritical fluid chromatography (SFC) using different mixtures. Their selectivity was compared to the commonly used columns in LC and SFC, namely octadecyl silicagel (C18) and 2-ethylpyridine, respectively.

SAMENVATTING

Gedurende de laatste jaren is er wereldwijde bezorgdheid over de toenemende distributie van hormoonontregelaars in het milieu. Deze angst wordt veroorzaakt door het nadelige effect dat deze stoffen hebben op het endocrien system van mensen en dieren, zelfs bij zeer lage concentraties zoals 1 ng/L. De invloed van deze EDCs op het voortplantingssysteem van dieren is reeds grondig onderzocht, maar de effecten op de mens zijn nog steeds het onderwerp van intens debat.

Hoofdstuk 1 geeft een kort overzicht van het mechanisme van de hormonale verstoring en de effecten op mens en dier. Verder, worden ook verschillende manieren waarop de mens wordt blootgesteld aan EDCs besproken, samen met de verschillende mogelijke methoden voor de bepaling van EDCs in waterige monsters.

Hoofdstuk 2 bespreekt de bepaling van EDCs in waterige monsters. De klasse van EDCs is niet alleen chemisch zeer heterogeen, maar de matrix waarin de EDCs voorkomen in het milieu is ook nog is zeer complex. Verder moet de methode in staat zijn om de EDCs zeer gevoelig te bepalen op de lage concentraties waarin ze voorkomen in waterige monsters. Daarom is pre-concentratie voor de analyse noodzakelijk. In dit hoofdstuk wordt een overzicht gegeven van de meest gebruikte monstervoorbereidingstechnieken in waterige monsters en hun toepassing voor de bepaling van EDCs wordt geillustreerd.

Hoofdstuk 3 beschrijft de ontwikkeling van een multi-residue methode voor de bepaling van EDCs in waterige monsters. Roervlo sorptieve extractie (SBSE) werd eerst gebruikt voor de bepaling van pyrethroïden in water om zo de verschillende desorptietechnieken te evaluaren. Thermische desorptie werd uitgevoerd in een klassieke split-splitless inlaat gemodificeerd met een flip-top systeem en een conventionele thermische desorber. Deze twee desorptietechnieken werden dan vergeleken met vloeistofdesorptie met ethylacetaat. Verschillende parameters die een invloed hebben op de extractie en de desorptie werden geoptimaliseerd. De hoogste gevoeligheid werd bereikt wanneer desorptie werd uitgevoerd in de conventionele thermische desorber. Daarom werd deze desorptietechniek gekozen voor de mulit-

residue methode. Vier verschillende monstervoorbereidingsprocedures werden in parallel uitgevoerd op vier delen van hetzelfde watermonster. Drie in-situ derivatisatiereacties specifiek voor fenolen, amines en zuren, en de organotinverbondingen werden uitgevoerd op drie delen van het watermonster. Aan het vierde deel werd methanol toegevoegd om zo de glasadsorptie van zeer apolaire verbindingen te verhinderen. Na de extractie werden de vier roervlo's in één glazen desorptebuisje geplaatst. Tijdens de thermische desorptie werd in-tube silylatie uitgevoerd met BSTFA, gevolgd door analyse via GC-MS. De methode werd geëvalueerd met een standaardmengsel van EDCs. Nadien werd de bruikbaarheid van de methode onderzocht door de analyse van een aantal reële watermonsters.

De extractie van polaire analieten uit waterige monsters is nog steeds beperkt. In *Hoodstuk 4*, werden twee strategiën onderzocht die de extractie van polaire analieten zou kunnen verbeteren. De eerste strategie omvat de ontwikkeling van een nieuwe sorptieve extractietechniek namelijk silicone membrane sorptive extraction (SMSE). Een PDMS buisje wordt gevuld met een organisch solvent en in de waterige oplossing geplaatst. Na de extractie wordt het organisch solvent geanalyseerd via GC-MS of LC-MS. De extractie werd geoptimaliseerd voor de bepaling van atrazine en zijn polaire metabolieten. Nadien, werd de bruikbaarheid van SMSE geëvalueerd via de analyse van een complex mengsel van EDCs and farmaceutische componenten. In het tweede deel van dit hoodstuk wordt een monolithisch materiaal gebruikt als extractiemiddel. De bruikbaarheid van dit materiaal werd onderzocht door headspace analyse van koffie. Het is onmogelijk om dit materiaal te gebruiken als coating op een roervlo omdat de robuustheid onvoldoende is.

In *Hoofdstuk 5* werd een bibliotheek van mogelijke mimics van de oestrogeenreceptor gesynthetiseerd. De affiniteit van zijn leden voor 17-β-estradiol werd geëvalueerd via twee screening technieken. De eerste is gebaseerd op affiniteitschromatografie. Affiniteitskolommen werden bereid door 17-α-ethinyl estradiol of 19-norethindrone te immobiliseren op de stationaire fase. De tweede screening technique is gebaseerd op vaste fase extraction. De extractie-efficientie van twee leden van de bibliotheek werd vergeleken met die van een commercieel

beschikbaar Oasis HLB® vaste fase materiaal voor de analyse van kunstmatig gepollueerd laboratorium water en afvalwater van een ziekenhuis. Op basis van de beschikbare literatuur, werden zeer belovende resultaten verwacht. Spijtig genoeg, zijn de besckibare technieken niet succesvol.

Hoofdstuk 6 bespreekt de selectiviteit van de kolommen die gemaakt zijn voor de affiniteitsstudie via verschillende mengsels in zowel omkeerfase LC als in SFC. Hun eigenschappen worden vergeleken met conventionele kolommen in LC en SFC namelijk octadecyl silicagel (C18) and 2-ethylpyridine, respectievelijk.

SCIENTIFIC PUBLICATIONS

Towards automated, miniaturized and solvent-free sample preparation methods
F. David, E. Van Hoeck and P. Sandra
Analytical and Bioanalytical Chemistry, Volume 387, Issue 1, 2007, Pages 141-144

Stir bar sorptive extraction for the determination of pyrethroids in water samples: A comparison between thermal desorption in a dedicated thermal desorber, in a split/splitless inlet and by liquid desorption
E. Van Hoeck, F. David and P. Sandra
Journal of Chromatography A, Volume 1157, Issues 1-2, 2007, Pages 1-9

Determination of fluoxetine in plasma by gas chromatography-mass spectrometry using stir bar sorptive extraction
C. Fernandes, E. Van Hoeck, P. Sandra and F.M. Lanças
Analytica Chimica Acta, Volume 614, Issue 2, 2008, Pages 201-207

Multi-residue screening of endocrine disrupting chemicals and pharmaceuticals in aqueous samples by multi-stir bar sorptive extraction-single desorption-capillary gas chromatography/mass spectrometry
E. Van Hoeck, F. Canale, C. Cordero, S. Compernolle, C. Bicchi and P. Sandra
Analytical and Bioanalytical Chemistry, DOI 10.1007/s00216-008-2339-7

Determination of atrazine and its metabolites in aqueous samples using silicone membrane sorptive extraction (SMSE) followed by GC-MS and LC-MS
E. Van Hoeck, E. Dumont and P. Sandra
Submitted to Chromatographia

A comparison between molecularly imprinted polymers and traditional sorbents for solid-phase extraction. Application to the extraction of triazines from aqueous samples and quantification by GC-MS

V.Malanchin, F. Lynen, E. Van Hoeck and P. Sandra

Submitted to Chromatographia

Towards a new SPE material for EDCs: Fully automated synthesis of a library of tripodal receptors followed by a fast screening via affinity LC.

S.E. Van der Plas, E. Van Hoeck, F. Lynen, P. Sandra and A. Madder

Submitted to European Journal of Organic Chemistry

Click chemistry used as an efficient strategy for the manufacturing of dedicated stationary phases for LC and SFC

E. Van Hoeck, S. Van der Plas, M. Dunkle, F. Lynen. A. Madder, P. Sandra

In preparation